CLXIV. THE PHOTOCAPILLARY REACTION OF PLANT SAP.

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Experiments have been carried out on the phenomenon which can be observed when the sap expressed from etiolated seedlings is suddenly illuminated. The surface tension of the sap increases until an equilibrium is attained. This depends on various factors and is destroyed in the dark so that the surface tension decreases.

It cannot be *a priori* stated whether this phenomenon belongs to the photochemical reactions as commonly understood; on the other hand, it is not entirely excluded that the changes of surface tension are brought about by physical forces, so that it is advisable to call the phenomenon a *photocapillary reaction of plant sap.*

The photocapillary reaction was first preliminarily described by the author in a paper on the influence of light on plants in relation to the surface tension of their sap [Herčík, 1926]. It was there stated that etiolated plants give a sap which has low surface tension and which is therefore rich in capillary-active substances. After illumination the amount of capillary-active substances in a plant is more and more reduced so that the plant gives a sap with a higher surface tension. Light therefore increases the surface tension in the plant so that the plant carries out a photocapillary reaction which can be observed more easily *in vitro* if only the expressed sap of etiolated plants is used instead of the plant itself. The sap is found to have a reactivity of the same order as that of the intact plant itself.

Methods.

Photocapillary reaction was observed in the sap expressed from young etiolated seedlings of *Pisum*, *Vicia*, *Pharbitis* and *Sinapis*. The reaction of *Sinapis* sap being the most intense, young *Sinapis* seedlings were chosen for experimentation.

Seedlings of *Sinapis alba* were cultivated on thick layers of filter-paper in the dark at 20°. Before the experiment they were cut into small pieces and ground in a porcelain mortar with fine sand to a dense pulp. This pulp was pressed out and the sap collected in small watch-glasses with a diameter of 5 cm. The

surface tension was measured with the Du Noüy tensiometer, 2 cc. of the sap being used for each measurement. The sap was then illuminated with a strong Osram-Nitra lamp, 500 w., 110 v., from different distances.

The static surface tension of the sap was very soon attained, the sap therefore was illuminated directly after squeezing. The diluted sap also very soon attains its static surface tension which, however, can be very easily disturbed. Therefore several watch-glasses (ten or more according to the number of measurements) were exposed to the light, each glass being used for only one single measurement, so that the surface of the sap was not disturbed by repeated measurements.

Experiments.

The experiments were made with sap alone and with sap which had been diluted to various extents with distilled water. The intensity of illumination amounted to 1356 lux.

Table I gives the photocapillary reaction of undiluted sap after illumination with 1356 lux. t' represents time in minutes after exposure to the light and σ surface tension in dynes per cm.

Table I.

Exp. no.	Age of the plants (days)		Changes of surface tension after illumination with 1356 lux										
79	7	t'	0	3	5	7	9	12	17	22	32	52	62
		σ	40·8	43·2	44·4	45·3	46·0	47·1	48·5	47·4	48·0	47·4	47·1
99 *a*	9	t'	0	12	19	27	46	60					
		σ	46·4	46·1	46·8	46·5	46·2	46·1					
93	12	t'	0	5	10	20	25	30	45	60	85		
		σ	38·4	44·3	45·4	45·8	46·3	46·4	47·0	46·4	46·4		
101	16	t'	0	5	10	16	22	34	45	60	121		
		σ	37·2	37·2	39·0	40·3	41·5	42·5	44·1	44·1	44·8		

On Fig. 1 is plotted the reaction curve of Exp. 79. It can be seen that equilibrium is reached after a number of small oscillations. The whole rise of surface tension which the light induces amounts here to 7·7 dynes per cm., in Exp. 99 *a* 0·4 dynes per cm., in Exp. 93 8·3 dynes per cm., and in Exp. 101 7·6 dynes per cm.; the effect which light brings about is therefore comparatively large. The deviation in the case of Exp. 99 *a* is due to the fact that here the initial surface tension of the sap differs greatly from that in the other experiments. It follows that a certain minimum surface tension is necessary for the reaction to begin at all. From other experiments which are referred to in another place [Herčík, 1927, 2] it can be deduced that this minimal surface tension corresponds to about 44·2 dynes per cm.

Control experiments were also made to find out if the sap does not change its surface tension spontaneously in the dark. Table II, in which are recorded some of these experiments, shows that in the dark no change which could be taken into consideration occurs.

Table II.

Age of the plants (days)		Changes of surface tension in the dark			
5	t'	0	20	30	50
	σ	47·0	47·7	47·7	48·3
10	t'	0	42	102	132
	σ	40·5	40·8	41·5	41·9

The photocapillary reaction of diluted sap is different from that of undiluted sap. The surface tension decreases instead of increasing (Table III).

Table III.

Exp. no.	Age of the plants (days)	Concentration of the sap		Changes of surface tension after illumination with 1356 lux					
99 *b*	9	80%	t'	0	11	17	26	45	59
			σ	46·2	45·4	48·9	47·2	45·3	45·1
99 *c*	9	60%	t'	0	9	10	25	44	58
			σ	46·8	45·6	47·8	45·0	43·4	43·3
99 *e*	9	20%	t'	0	6	14	23	42	56
			σ	51·8	48·6	48·3	48·6	47·5	47·8
99 *f*	8	10%	t'	0	5	13	22	41	55
			σ	51·1	48·1	47·1	46·4	45·6	44·9

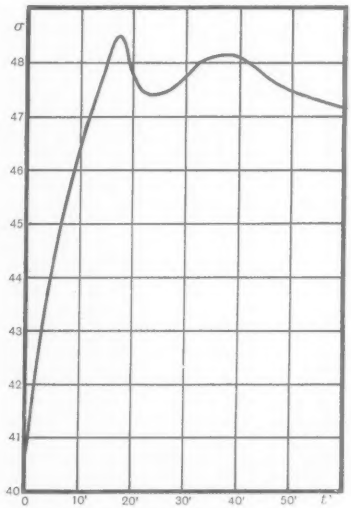

Fig. 1.

From the table it can be seen firstly, that the photocapillary reaction of diluted sap is *in nuce* a reversed photocapillary reaction of concentrated sap, *i.e.* the surface tension decreases; and secondly, that the mode and extent of this decrease depend on the degree of dilution.

In 80 % dilution the surface tension at first decreases a little, then strongly increases and again decreases. In 60 % dilution the first increase is smaller and on the contrary the second decrease is more intense. The 20 % sap shows practically no increase, the surface tension decreasing here after illumination

almost regularly. The 10 % sap finally gives a typical negative photocapillary reaction: the surface tension here decreases in a smooth parabolic curve (Fig. 2).

The reaction of slightly diluted sap is therefore composed of both types of photocapillary reaction, of a positive and negative type, indicated by rise and fall of surface tension. The predominance of one of these two types is given by the degree of the dilution. At higher dilutions the negative reaction takes place, at lower dilutions the positive reaction occurs. For other types of the reactions of the diluted sap see the previously mentioned paper of the author [Herčík, 1927, 2].

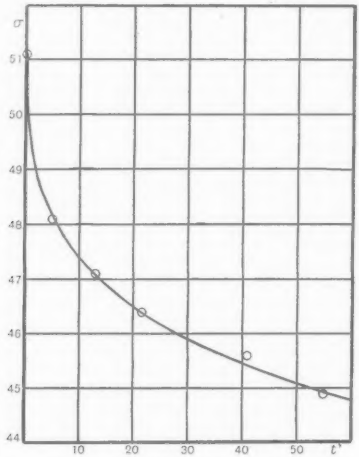

Fig. 2.

In Table IV control experiments are recorded from which we see that the surface tension of diluted sap does not change when the sap remains in the dark.

Table IV. Sinapis alba, 10 *days old etiolated seedlings. Changes of surface tension of sap, diluted to various degrees, in the dark.*

t'	50 %	25 %	12·5 %
0	49·3	51·8	53·7
53	49·7	51·4	53·5

The question naturally arises whether the photocapillary reaction depends in any way on the intensity of the light used. Three different illuminations were taken into consideration, namely of 11,720 lux, 5,420 lux and 13·19 lux. The experiment with the illumination of 11,720 lux is given in Table V.

Table V. Sinapis alba, 12 *days old etiolated seedlings.*

Conc. of the sap	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Darkness	44·5	47·4	51·9	51·8	53·8	56·4	63·0	65·3	70·2
Light 60′	48·6	45·8	48·7	50·0	52·2	54·3	58·1	58·3	60·2
Darkness 7½ hours	45·8	45·8	46·3	48·6	50·8	56·3	55·5	60·8	65·0

In this experiment the surface tension of the sap in the dark was first measured. Then the sap was illuminated with 11,720 lux for 1 hour. The surface tension of the undiluted sap increased (only a little because the initial surface tension of the sap was high); that of the diluted sap decreased (in the dilution 1/32 there was no change). After this illumination, the samples were kept in the dark for 7½ hours altogether. This caused a decrease of surface tension of undiluted sap and of the diluted sap in the lower dilutions. In the higher dilutions the surface tension increased.

This shows that with negligible deviations the photocapillary reaction has a reversible character.

On the other hand, a quantitative effect of the higher illumination on the amount of the reaction cannot be observed. The same was established when illuminations of 5420 and 13·19 lux were used. The amount of the reaction in both cases was nearly the same. [See for further detail Herčík, 1927, 2.]

Discussion.

It cannot be maintained that this photocapillary reaction is a photochemical reaction as is generally understood. It is quite independent of the intensity of the light used, it has no induction period and only two decisive factors, namely, the initial surface tension of the sap and the degree of dilution.

The fact that the photocapillary reaction cannot be brought about when the initial surface tension of the sap is relatively high, points to the conclusion that the photoactive substances are highly capillary-active, *i.e.* they determine the lowest limit of the surface tension of the sap.

The degree of dilution strikingly changes the character of the reaction, the positive reaction being converted into a negative one bringing about a decrease of surface tension.

It is reasonable to suppose that the photocapillary reaction is governed by the Hallwachs photoelectrical effect. This suggestion is supported by the following facts. The living substance is known to carry a negative charge. The particles of the sap are undoubtedly also negatively charged. As is known every charge diminishes the surface tension, so that the surface tension of the living substance and also of the sap is reduced according to the amount of the charge present on single particles.

Zwaardemaker and Hogewind [1919] state that the Hallwachs phenomenon can be largely applied for colloidal solutions and therefore also for living substance. This means that the living substance and the sap probably lose their negative charge after illumination and the surface tension rises until an equilibrium is attained. The negative photocapillary reaction can also be explained on the basis of the photoelectrical effect, supposing that the particles lose their charge when the sap is diluted. They are therefore made electroneutral and may be again charged by the action of the light [see Righi, 1888]. The surface tension in this case decreases.

The positive photocapillary reaction which is also carried out by green plants appears to be one of the most important factors in the growth-response of the plants to light. The growth-retarding action of the light can be more easily understood in the following way: by means of photocapillary reaction light increases the surface tension on interfacial surfaces of the cell, and this increased surface tension retards the growth. In the dark the process occurs in the reverse way, the surface tension is reduced and growth is thereby favoured [see Kříženecký, 1927; Bauer, 1924; Kopaczewski, 1924; Herčík, 1926, 1927, 1].

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CLXV. STUDIES ON XANTHINE OXIDASE. IX.

THE SPECIFICITY OF THE SYSTEM. II.

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One of the most remarkable phenomena connected with enzyme action is the very strict specificity of many enzymes for their substrates.

The oxidising enzymes have not yet been thoroughly studied from this point of view, and an enzyme which lends itself especially well to an investigation of this kind is the xanthine oxidase. The enzyme is easily obtained free from other enzymes and the purine group is particularly well adapted to this study because of the very large number of small changes that can be made in it.

Morgan, Stewart and Hopkins [1922] showed that this enzyme could activate both hypoxanthine and xanthine. They also showed that caffeine (1.3.7-trimethylxanthine), theobromine (3.7-dimethylxanthine), uric acid and guanine (2-aminohypoxanthine) were not activated by the enzyme, but they observed that adenine (6-aminopurine) was slowly oxidised under its influence. They ascribed the action on this last substance to a previous deamination of the adenine to hypoxanthine by some adenase that was present and the subsequent oxidation of the hypoxanthine to uric acid. Dixon and Thurlow [1924] thought, however, that it was probable that there was a direct action of the xanthine oxidase on the adenine and brought some evidence in support of this theory. Following up the above results Dixon [1926] investigated the action of the enzyme on 35 substances of physiological importance, but except for the above-mentioned substances and aldehydes the results were entirely negative.

The enzyme is a particularly interesting one because we can study separately the adsorption of the substrate on the enzyme and also the activation of the substrate by the enzyme, since Dixon and Thurlow [1924] have shown that those purines that are adsorbed inhibit the reduction of methylene blue by hypoxanthine or xanthine, owing to the formation of an interfering film on the surface of the enzyme. Activation of the purine can thus be detected by observing whether the methylene blue is reduced, and, on the other hand, adsorption can be studied by observing the effect of the purine on the velocity of the reduction of the dye by the xanthine.

Thus, by a study of a series of substituted purines, we may hope to determine what structure is necessary for (*a*) activation, and (*b*) adsorption of the substrate.

Effect of structure on activation.

The following purines were tested:

3-methylxanthine	3.8-dimethylxanthine
8-methylxanthine	1-methylguanine
9-methylxanthine	7-methylguanine
1.3-dimethylxanthine	1.7-dimethylguanine

and also benziminazole.

The whey preparation of Dixon and Kodama [1926] was used as a source of the enzyme and the results were confirmed using fresh milk. The experiments were carried out in Thunberg tubes in the usual way. The tubes were always evacuated, washed out with nitrogen and re-evacuated before being placed in the bath. The method used in the experiments was as follows.

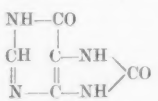
0·5 cc. fresh milk, 5 cc. buffer, 0·5 cc. methylene blue (1 in 1000) and 0·1 cc. of a solution of xanthine containing 5 mg. per cc. were placed in the bath at 40°; decoloration was complete in 120 secs. 2 cc. of the same milk, 3 cc. buffer, 0·5 cc. of the methylene blue and 0·1 cc. of a solution of the purine to be tested containing 5 mg. per cc. were also placed in the bath.

In no case of the above series of purines was there found to be any visible change of colour. Since a change of one quarter of the colour should be readily detected and the observations extended over 4 hours it is easily calculated that, if any of the purines were activated, the velocity of the reaction must have been less than one two-thousandth of that with xanthine.

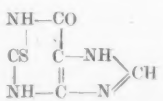
In the above experiment all the solutions of the purines were made up in the following manner. 50 mg. of each was accurately weighed into a 10 cc. graduated vessel. About 2 cc. of water was added and a drop of strong caustic soda. The vessel was agitated and when solution was complete water was added to the mark. Just before use very small quantities of strong hydrochloric acid were added until a cloudiness was apparent and this was just cleared by the addition of minimal amounts of dilute caustic soda. In this way the purines were obtained in a solution which was not alkaline enough markedly to affect the p_H of the reaction mixture.

The significance of the results is discussed later.

Two purines, other than hypoxanthine, xanthine and adenine, have been found which are activated by the enzyme, namely:



6.8-Dihydroxypurine



2-Thioxanthine

0·5 cc. fresh milk, 5 cc. methylene blue (1 in 1000) and 0·1 cc. of the solution of the purine containing 5 mg. per cc. were taken in each experiment.

	Time for complete reduction (secs.)
Hypoxanthine	120
Xanthine	225
6.8-Dihydroxypurine	250
2-Thioxanthine	350

From a consideration of their structure it might be expected that one molecule of the last two purines would reduce one molecule of methylene blue, since they would, in all likelihood, be oxidised to uric acid and thiouric acid respectively. To test this the following technique was devised and very clean-cut results were obtained. 0·15 cc. quantities of the solution of the purine to be tested (5 mg. per cc.) were accurately measured by means of a Pregl pipette into small test-tubes and these were introduced into large Thunberg tubes, which contained 10 cc. of milk and the various under-mentioned amounts of methylene blue. The Thunberg tubes were then evacuated several times, being washed out with nitrogen each time, and when this was complete the tubes were tilted so that the purine was mixed with the milk and the methylene blue, and the tubes were placed in the bath at 40° or 60°. It was found that, when using the 6.8-dihydroxypurine, 4·3 cc. of methylene blue (M/1000) were reduced very quickly, 4·5 cc. almost as quickly but that with 4·7 cc. a slight colour persisted for a considerable time. Thus the purine required about 4·6 cc. of the methylene blue and this is in good agreement with the theoretical figure of 4·9 cc. With the thioxanthine, 4·3 cc. was obtained for the titration instead of 4·45 cc. The agreement is excellent and the slight disparity is probably due to unavoidable traces of oxygen.

Xanthine oxidase prepared from ox spleen by the method of Morgan, Stewart and Hopkins was also capable of bringing about the oxidation of these purines. Using about 5 cc. of spleen, 0·5 cc. of methylene blue (1 in 1000) and 0·1 cc. of the purines (5 mg. per cc.) decoloration was complete in the following times.

At 40° (control without purine about an hour):

6.8-Dihydroxypurine	90 secs.
2-Thioxanthine	140

At ordinary temperatures (control without purine more than 8 hours):

Hypoxanthine	4·5 mins.	(4·8)
Xanthine	9	(9)
6.8-Dihydroxypurine	10	(10)
2-Thioxanthine	15	(14)

The numbers in the brackets are calculated from the figures given above when using milk, taking xanthine as the standard of reference. The satisfactory agreement of the velocity ratios shows that the same enzyme is responsible for the activation of all four purines. It is hoped to isolate the products of the oxidation of the 6.8-dihydroxypurine and the 2-thioxanthine.

Effect of structure on adsorption.

In order to determine which of the purines were adsorbed by the enzyme a series of experiments was carried out on each of them. Using a constant

amount of xanthine and adding varying quantities of the purine to be tested, inhibition curves were plotted for each purine and these are given graphically (Fig. 1). The exact description of the experiments is as follows. 2 cc. of buffer, 2 cc. of enzyme (2 % whey preparation), x cc. of the purine solution (5 mg. per cc.) and $(2 - x)$ cc. of water were placed in a series of Thunberg tubes. When the series was ready 0·1 cc. of xanthine solution (also 5 mg. per cc.) was quickly added to each, the tubes evacuated as quickly as possible, re-evacuated and placed in the bath at 40°. The time for the complete decoloration of each tube was noted and this was converted into speed by multiplying its reciprocal by 10,000, according to the procedure of Dixon and Thurlow, who also give other inhibition curves.

Discussion.

The results obtained in the experiments on the effect of structure on activation show in a striking manner the high degree of specificity which obtains in this system. The introduction of a single methyl group in either ring is sufficient entirely to prevent any activation, although the inhibition curves show that the enzyme still adsorbs the compounds, but not so strongly as the unsubstituted xanthine. Dixon [1926] showed that the introduction of an amino-group in the 2 position was also sufficient completely to prevent any action, and the introduction of an amino-group in the 6 position (adenine) reduced the velocity to one-fiftieth of that of xanthine.

The fact that 6.8-dihydroxypurine and xanthine are both converted to uric acid at approximately the same rate shows that the two stages of the oxidation take place independently of one another. Particularly interesting is the case of 2-thioxanthine, in which the place of one of the atoms of oxygen in xanthine is taken by an atom of sulphur. As far as the enzyme is concerned, it seems that the sulphur behaves practically in the same way as the oxygen. There is, however, a slight difference in the speed of oxidation—the sulphur compound being oxidised a little more slowly, but this may be due to the molecule not being adsorbed to the same extent.

In the inhibition experiments it seems that the purines can be divided into three classes:

(*a*) those strongly adsorbed:

Hypoxanthine	6.8-Dihydroxypurine
Xanthine	Guanine
3-Methylxanthine	1-Methylguanine
Uric acid	7-Methylguanine
Adenine	1.7-Dimethylguanine

(*b*) those adsorbed to only a small extent:

8-Methylxanthine	Alloxan
9-Methylxanthine	

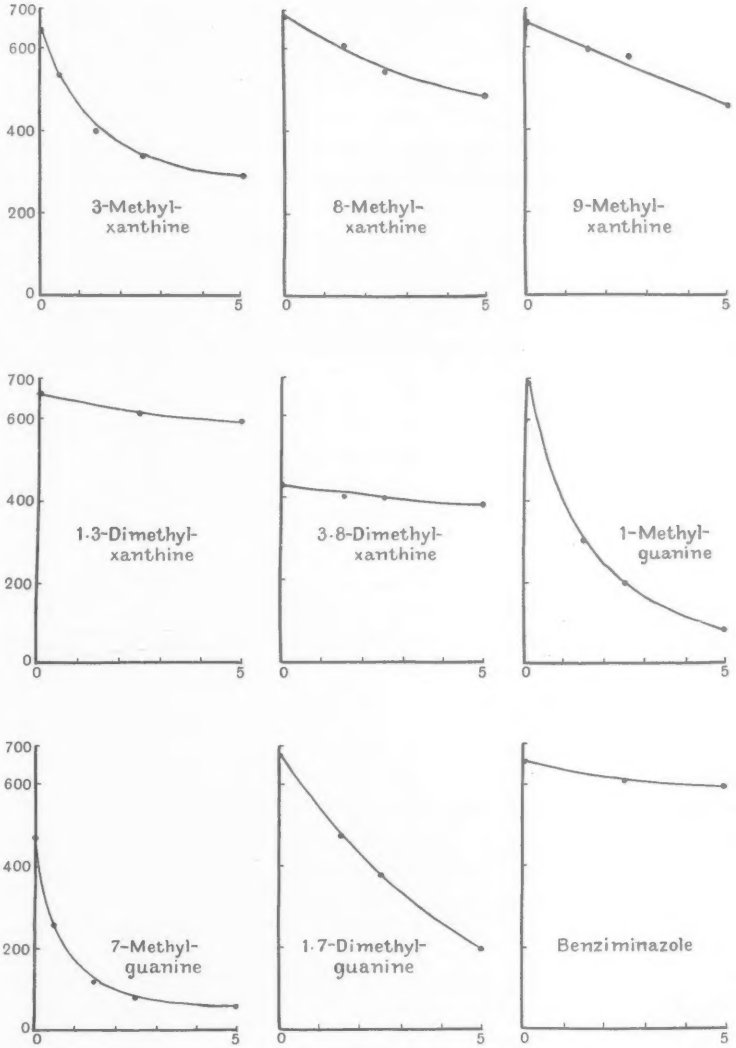


Fig. 1.

Ordinates represent reaction velocity. Abscissae give mg. of purine added

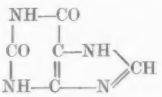
(*c*) those not absorbed, or only to a very slight extent:

Benziminazole	Thymine
1.3-Dimethylxanthine	Cytosine
3.8-Dimethylxanthine	Uracil
Caffeine (1.3.7-trimethylxanthine)	Histidine
Theobromine (3.7-dimethylxanthine)	

(This table also includes the results of other workers.)

It is clear that neither the pyrimidine nor the iminazole ring can by itself cause adsorption of the enzyme—it seems necessary to have the complete purine structure. The pyrimidines are not adsorbed, neither is an iminazole compound such as histidine, and the same is true of benziminazole which contains a benzene ring instead of the pyrimidine ring which is present in the purines. On the other hand, the iminazole ring seems to be very necessary for adsorption and this is very clearly demonstrated by a comparison of uracil

Uracil



Xanthine

and xanthine, because, whereas the latter is strongly adsorbed the former is not.

The introduction of a methyl group, particularly in the iminazole ring, tends to prevent adsorption. Thus in the methylxanthines we have much less adsorption than with xanthine itself. The effect of methyl groups in preventing adsorption is very plainly shown when more than one is introduced—these compounds are only adsorbed to a very small extent.

The introduction of an amino-group strongly favours adsorption, so much so as to overpower the effect of the methyl groups. In the guanines the monomethyl compounds are adsorbed much less than guanine itself but even when two methyl groups are present adsorption is very marked, whereas in the case of the xanthines two methyl groups almost completely prevent adsorption.

It seems likely from the above results that the attachment of the substrate to the enzyme is due partly to the iminazole ring and also partly to the structure of the 1.6-position. The data at present available, interesting and suggestive as they are, are insufficient to afford a basis for a complete theory of the mechanism of adsorption, but it is hoped to complete the above series of experiments so that a satisfactory theory can be evolved.

Summary.

1. The action of the xanthine oxidase on a series of substituted purines has been studied with a view to determining the effect of structure of the purine on (*a*) its activation, (*b*) its adsorption by the oxidase.

2. The enzyme was able to activate only two of these purines, namely, 6.8-dihydroxypurine and 2-thioxanthine.

3. These two purines take up one atom of oxygen for each molecule during their oxidation by this system.

4. The enzyme is very specific, the introduction of a methyl group into the pyrimidine or iminazole ring being sufficient entirely to prevent activation.

5. The complete purine skeleton—the two-ring structure—is necessary for adsorption. The introduction of an amino-group strongly favours adsorption, and the introduction of methyl groups, particularly in the iminazole ring, tends to prevent adsorption.

The author is much indebted to Dr M. Dixon who suggested this line of work to him, and both to him and Mr E. J. Morgan for many suggestions, as well as to Dr Hermann Fischer, Dr H. W. Dudley and Messrs C. F. Böhringer who generously provided him with pure specimens of many of the compounds listed.

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CLXVI. STUDIES ON THE ANTINEURITIC VITAMIN.

I. PRELIMINARY NOTE ON A POSSIBLE SECOND FACTOR.

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(*Received September 13th, 1927.*)

Attention has been drawn by Plimmer and Rosedale [1926] and Plimmer [1926] to the difference in visible symptoms in pigeons on a total deficiency and a partial deficiency of vitamin B in the diet. While the typical paralysis of acute polyneuritis occurred in the former case, shortage in amount of vitamin B led to general debility, the time of appearance depending upon the amount of the vitamin in the ration. The visible effect of shortage is first observed by a falling off of the food consumption, the scattering of the food and the inability of the birds to fly up to the perches of the house. *Post mortem* examinations have shown conditions described by McCarrison [1921], one of the most noticeable being the presence of large masses of decomposing food material in the intestine.

During the course of the preparation of a strong extract of vitamin B from rice polishings a point of interest concerned with the difference between acute polyneuritis and the "shortage" of the vitamin has been observed.

1. Rice polishings have been extracted by 1 % acetic acid, 2 litres being used for each 500 g. of polishings. The extraction is carried on for 8 hours at 40° with constant stirring. After filtering, the extract is concentrated *in vacuo* at 40° to such volume that 1 cc. corresponds to 1 g. of the original polishings. 5 cc. of this extract suffice to cure acute polyneuritis within a few hours. 2 cc. daily, administered to pigeons receiving a diet composed solely of white polished rice, produce steady growth and secure complete maintenance of health and functions.

2. This first extract has been treated with lead acetate until no further precipitate could be obtained, when it has been filtered, the lead being removed by H_2S, which in turn has been expelled by boiling *in vacuo* at 40°. The filtrate and the precipitate after treatment with lead acetate have each separately been subjected to these operations, each being finally made to such volume that 1 cc. corresponds to 1 g. of polishings.

The filtrate portion behaves similarly to the first extract in that 5 cc. will cure acute polyneuritis and 2 cc. given daily will maintain the health of pigeons on a diet of white rice.

2 cc. of the precipitate portion, given daily, enable birds to maintain growth, health and natural functions. This portion, however, will not cure typical acute polyneuritis. The administration of this portion to birds suffering from polyneuritis causes rapid evacuation of the bowel, and, while death ultimately ensues, the usual "stoppage" symptoms are not found on *post mortem* examination. Birds which do not show the typical polyneuritis symptoms, but which have become enfeebled by white rice feeding, are sufficiently relieved by the administration of 5 cc. of this fraction to be able to feed and to remount the perches a few hours after the dose.

In view of this fact, and also on account of the observation that the health of pigeons could be maintained on small quantities, it was thought that small portions of vitamin B had been precipitated by the lead acetate. An amount of this extract corresponding to 2 kg. was concentrated *in vacuo* at 40° and administered in a dose of 20 cc. to a polyneuritic pigeon without cure being effected. This concentration and dosing has been carried out seven times with the same result.

It appears, therefore, that two factors are concerned in the ordinary rôle of vitamin B, one of which has to do with digestive processes, but is independent of the anti-paralysis factor, and is precipitated by lead acetate.

Further chemical and clinical investigations are in progress.

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CLXVII. THE EFFECTS OF COD-LIVER OIL ON THE CALCIUM AND PHOSPHORUS METABOLISM OF THE LACTATING ANIMAL.

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(*Received September 14th, 1927.*)

The demands of the modern dairy animal for calcium and phosphorus are great. The desirability of maintaining a positive balance of these elements has been emphasised recently by Orr [1923], Erf [1923] and Henderson and Magee [1926]. Large amounts of these minerals leave the body in the milk and, where pregnancy and lactation are concurrent, the requirements of the foetus must also be supplied if both the cow and her calf after birth are to be maintained in good condition. The retention of adequate amounts of these minerals is thus a matter of physiological as well as of economic importance. Unfortunately in modern practice the feeding of concentrated foodstuffs, while supplying an abundance of the organic constituents of the diet, will not ensure a sufficient supply of the inorganic elements, since their content of these is, in general, low.

Cod-liver oil is a well-recognised stimulant of calcium and phosphorus assimilation as has been shown by McCollum *et al.* [1922] with rats and by Husband, Godden and Richards [1923] with growing pigs. It therefore seemed of interest to determine whether cod-liver oil would affect the calcium and phosphorus metabolism of the lactating animal in the same way. With that object in view, the following experiments were carried out.

Methods.

Animals. Goats were used. The procedure was similar to that described by Henderson and Magee [1926].

Feeding. The food consisted of two portions, one of grain and one of hay, the former being made up from a mixture of the following amounts of cereals: 72 lb. ground maize, 28 lb. oatmeal, 14 lb. linseedmeal.

Before the experiment began the animal was fed to satisfaction under the conditions of the experiment and, at the end of a week, a knowledge of its appetite had been obtained. Suitable amounts of grain and hay were then decided upon and the ration was kept at this level throughout the experiment. During the experimental period the oil was mixed with the grain ration and

fed in two approximately equal parts in the morning and evening. Distilled water was given *ad lib.* in each experiment.

As far as possible, each experiment was divided into three periods of 16 days' duration, the excreta being collected daily and analysed every 4 days by the following methods:

calcium: precipitation as oxalate and titration with $N/10$ potassium permanganate.

phosphorus: the Pemberton-Neumann method as modified by Richards and Godden [1924].

iodine: a modification by Leitch and Henderson [1926] of Fellenberg's titrimetric method.

milk-fat: ether extraction in a Soxhlet apparatus.

Exp. 1. The experiment began 257 days after parturition and was limited to two periods, a pre-period of 16 days and an experimental period of 20 days, there being no post-period owing to the onset of oestrum.

Diet: 450 g. grain and 900 g. hay per day containing 5·93 g. CaO and 6·39 g. P_2O_5. During the experimental period 10 cc. cod-liver oil were given daily.

Results:

Average daily excretion and balance in g.

	Weight of faeces	Output of CaO				Balance of CaO
		Milk	Faeces	Urine	Total	
Pre-period	1223	1·26	3·65	0·24	5·15	+0·78
Experimental period	1080	1·43	3·20	0·35	4·98	+0·95
		Output of P_2O_5				Balance of P_2O_5
Pre-period	—	2·40	3·84	0·05	6·29	+0·10
Experimental period	—	2·38	3·69	0·04	6·11	+0·28

During the oil-period there was an increased retention of calcium in spite of the increased output in both milk and urine. This was accounted for by the decreased output in the faeces, the average daily weight of which was reduced during the oil-period. The balance of phosphorus was also increased, but this was due to diminished excretion in milk and urine, as well as in the faeces.

The average daily milk volume was 550 cc. in the pre-period and 560 cc. in the oil-period. The percentage of calcium (Fig. 1) showed a definite increase immediately after the introduction of the oil, while the phosphorus and fat contents fell as the volume increased.

Exp. 2. The experiment began on the 23rd day of lactation. There were three periods, each of 16 days' duration.

Diet: 300 g. grain and 900 g. hay per day containing 8·49 g. CaO and 6·19 g. P_2O_5. During the experimental period 10 cc. cod-liver oil were given daily.

Results:

Average daily excretion and balance in g.

	Weight of faeces	Output of CaO				Balance of CaO
		Milk	Faeces	Urine	Total	
Pre-period	1066	1·79	7·19	0·05	9·03	−0·54
Experimental period	995	1·46	6·95	0·05	8·46	+0·03
Post-period	1063	1·24	7·14	0·05	8·43	+0·06
		Output of P_2O_5				Balance of P_2O_5
Pre-period	—	2·38	4·01	0·02	6·41	−0·22
Experimental period	—	1·80	4·74	0·02	6·56	−0·37
Post-period	—	1·56	4·57	0·02	6·15	+0·04

The balance of calcium showed a marked improvement during the oil-period because of reductions in the amounts in milk and faeces. In the post-period the faecal excretion increased, but the amount in the milk continued to fall with the result that the balance became more positive. There was no corresponding improvement in the phosphorus balance in the oil-period for here the faecal excretion reached its maximum. In the post-period the amounts in the milk and faeces fell and the balance became positive. The average daily weight of faeces was reduced during the oil-period and rose again in the post-period.

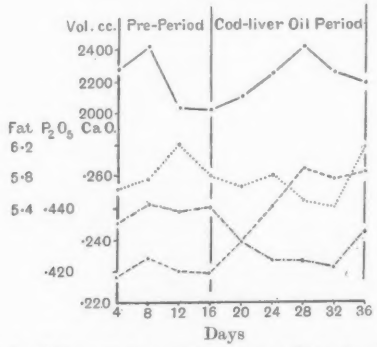

Fig. 1. Volume and percentage composition of milk.

——•—— Vol. ..... Fat

–•–•–•– P_2O_5 – – – – – CaO

The milk volume fell throughout the experiment, but the percentages of calcium and fat showed little variation except for a slight fall in the case of calcium in the post-period. The phosphorus content fell definitely from an average of 0·242 % in the pre-period to 0·223 % and 0·222 % in the oil- and post-periods respectively.

Exp. 3. The goat had been lactating for 162 days when the experiment began. There were two periods of 16 days and one of 18 days. The extension of the post-period was necessary because the animal developed diarrhoea and the intake was reduced by making the first two analyses over 5-day periods

and giving only 4 days' food. The figures for these 5-day collections were not used in calculating the average excretions and balances.

Diet: 750 g. grain and 1100 g. hay per day containing 9·93 g. CaO and 9·91 g. P_2O_5. During the experimental period 15 cc. of cod-liver oil were given daily.

Results:

Average daily excretion and balance in g.

	Weight of faeces	Output of CaO				Balance of CaO
		Milk	Faeces	Urine	Total	
Pre-period	1782	1·15	8·95	0·92	11·02	−1·09
Experimental period	1405	1·19	7·80	0·71	9·70	+0·23
Post-period	1488	0·92	7·73	0·26	8·91	+1·02
		Output of P_2O_5				Balance of P_2O_5
Pre-period	—	2·06	9·12	0·04	11·22	−1·31
Experimental period	—	2·03	7·87	0·04	9·94	−0·03
Post-period	—	1·80	6·56	0·02	8·38	+1·53

In the pre-period the balance of calcium was negative in each analysis, but after the introduction of the oil it became positive with one exception. The diminution in excretion occurred in the faeces, the average daily weight of which again showed a reduction in the oil-period. The balance of phosphorus showed a similar improvement in the oil-period. In the post-period the diarrhoea produced large negative balances of both minerals and the succeeding analyses showed positive balances.

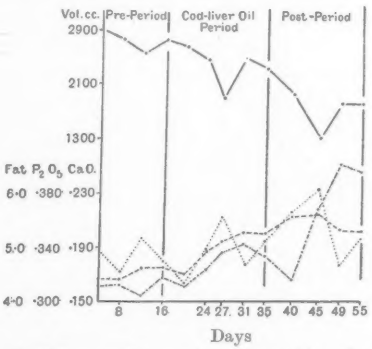

Fig. 2. Volume and percentage composition of milk.

——•—— Vol. ..... Fat
–•–•–•– P_2O_5 – – – – – CaO

During the oil-period the milk volume was slightly lower than in the pre-period, and in the post-period it was much more so owing to the diarrhoea (Fig. 2). When this passed off the volume rose, but never regained its former level. In the oil-period the percentage of calcium increased steadily, but reached its maximum in the post-period when the volume was at its minimum.

The phosphorus content also increased in the oil-period but, after a slight fall, rose much higher in the post-period. The percentage of fat showed wide variations and also reached its maximum in the post-period when the volume was lowest.

Exp. 4. The special objective of this experiment was to determine whether the increases in the calcium content of the milk in the oil-period of Exps. 1 and 3 were related to the stage of lactation. The animal was the same as in Exp. 1 and the experiment began on the 5th day of lactation. There were three periods of 16 days. Only the milk was analysed.

Diet: 500 g. grain per day with hay *ad lib.* 10 cc. of cod-liver oil were given daily during the experimental period.

Results: the average daily volume for the three periods was, pre-period 928 cc., oil-period 771 cc. and post-period 454 cc. The calcium content was unaffected by the oil, the average figures for the three periods being 0·220 %, 0·223 % and 0·265 %. The phosphorus percentage also showed little change during the oil-period: pre-period 0·335 %, oil-period 0·337 % and post-period 0·365 %. The average fat content was lowest in the oil-period, falling from 5·98 % in the pre-period to 5·56 % in the oil-period, and rising to 7·22 % in the post-period. It will be noted that all three constituents rose markedly in the post-period as the volume fell. In this experiment the iodine content was also estimated with the following results for each analysis:

Pre-period	13,	13,	13,	11γ %.	Av. 12·5,
Oil-period	17,	17,	17,	19γ %.	Av. 17·5,
Post-period	16,	15,	14,	13γ %.	Av. 14·5,

where $\gamma = 0{\cdot}000001$ g.

The average total amounts of iodine secreted in the milk per day were: pre-period, 116γ; oil-period, 135γ; post-period, 66γ. Thus the feeding of 10 cc. of cod-liver oil which contained 80γ of iodine increased both the percentage and the total iodine contents of the milk.

Exp. 5. Kelly [1925] has shown that with growing pigs the addition of small quantities of iodine as potassium iodide to the diet results in an increased retention of nitrogen and phosphorus, but has less definite effects on that of calcium. This suggested that the beneficial effects of cod-liver oil might be attributable to its high iodine content. An experiment similar to Exp. 3 was accordingly carried out in which the same animal was given an equal volume (15 cc.) of olive oil plus an equivalent amount of iodine (120γ) as potassium iodide. The experiment began on the 229th day of lactation; the pre-period and experimental periods were of 16 days' and the post-period of 12 days' duration.

Diet: 400 g. grain and 1200 g. hay per day containing 20·52 g. CaO and 9·85 g. P_2O_5. During the experimental period 15 cc. of olive oil and 120γ of iodine as potassium iodide were mixed with the grain every day. The large calcium intake was due to an unusual amount of clover in the hay.

Results:

Average daily excretion and balance in g.

	Weight of faeces	Output of CaO				Balance of CaO
		Milk	Faeces	Urine	Total	
Pre-period	1319	0·99	17·77	0·12	18·88	+1·64
Experimental period	1290	0·81	19·00	0·09	19·90	+0·62
Post-period	1243	0·59	20·35	0·08	21·02	−0·50
		Output of P_2O_5				Balance of P_2O_5
Pre-period	—	1·81	5·57	0·03	7·41	+2·44
Experimental period	—	1·41	8·39	0·03	9·83	+0·02
Post-period	—	0·89	9·50	0·03	10·42	−0·57

The balance of both calcium and phosphorus fell greatly during the experimental period as a result of greatly increased excretion in the faeces. The average daily weight of faeces fell throughout the experiment, but there were increases in the percentages of both calcium and phosphorus. In the post-period assimilation remained poor and the balances were, on the average, negative.

The milk volume fell continuously throughout from a daily average of 437 cc. in the pre-period to 326 cc. in the experimental period and 225 cc. in the post-period. During the experimental period the percentage of calcium rose steadily and remained high in the post-period, the average percentages being 0·227 %, 0·248 % and 0·264 % in the respective periods. The percentage of phosphorus also reached its maximum in the experimental period, the average figures for the period being 0·414 %, 0·434 % and 0·394 %. The fat percentage remained steady in the pre-period and in the first half of the experimental period, but rose to its maximum in the second half of that period and fell again in the post-period. The average iodine contents for the periods were: pre-period, 17γ %; experimental period, 23γ %; post-period, 22γ %. The average daily total iodine secreted in the milk was the same in the pre- and experimental periods, but fell markedly in the post-period.

Exp. 6. Exps. 1–4 indicated that the fat content of milk was not definitely affected by cod-liver oil in the amounts given, and in Exp. 5 the feeding of olive oil and potassium iodide gave an increased fat content in the second half of the experimental period. Further, the feeding of iodine in either organic or inorganic form produced definite increases in the iodine content of the milk. It was therefore decided to determine the effects of olive oil alone on the composition of the milk, in order to find whether the effects observed in Exps. 4 and 5, *viz.* increase in iodine and fat contents, could be attributed to the oil *per se*.

The animal used was the same as in Exps. 1 and 4 and the experiment was of the same length as Exp. 5. It began on the 69th day of lactation and only the milk was analysed.

Diet: 400 g. grain per day with hay *ad lib.* In the experimental period 15 cc. of olive oil were given per day.

Results: the milk volume rose throughout the experiment, the average daily figures being: pre-period, 537 cc.; experimental period, 605 cc.; post-period, 729 cc. The average percentages of calcium and fat were highest in the oil-period, but the average phosphorus percentage fell throughout; the average iodine content and total iodine were both at a minimum in the oil-period. These figures are given in the following table.

	CaO %	P_2O_5 %	Fat %	Iodine γ %
Pre-period	0·245	0·387	6·19	16
Experimental period	0·251	0·337	6·90	13
Post-period	0·234	0·362	6·33	14

Discussion.

Each of the cod-liver oil experiments shows a reduction in the average daily weight of faeces during the oil-period as compared with the pre- and post-periods. That this was not due to progressive constipation as a result of confinement is shown by the rise in the post-period in one case to within a few g. of the pre-period amount. This fall in the weight of faeces was accompanied by an increase in the percentage of calcium immediately after the introduction of the oil. In Exps. 2 and 3 the average percentage of calcium in the faeces was higher in the oil-period than in the pre-period and in Exp. 1 it fell by only a very slight amount.

In each of these experiments, however, there was an improved balance of calcium during the oil-period. In Exps. 1 and 3 this was completely accounted for by the reduction which took place in the faecal excretion; for in these there was an increased excretion in the milk while changes in the urinary excretion were comparatively slight. In Exp. 2 the amount of calcium in the faeces was also reduced, but the improved balance was not entirely due to this, for the amount in the milk was also falling.

This improved balance of calcium as a result of feeding cod-liver oil agrees with the work of Hart, Steenbock and Hoppert [1921], who found that with both lactating and dry goats the amount of calcium in the faeces was reduced.

The phosphorus balances, on the other hand, did not show a similar improvement. In Exps. 1 and 3 the increased retention was almost wholly the result of a reduction in the faecal excretion. The second experiment, however, showed no such improvement, as the faecal excretion was at its maximum in the oil-period; but it may be noted here that this animal was at a different stage of lactation from the others and showed other differing results.

The results with regard to calcium and phosphorus balances are not in agreement with those of Meigs [1926], who found no improvement in the retentions of either of these minerals or of nitrogen after feeding 100 cc. daily to cows.

Numerous experiments have been made in attempts to increase the concentration of a particular constituent of the milk by feeding excessive amounts of it in the diet, but these have generally failed [Lauder and Fagan, 1914;

Denis, Sisson and Aldrich, 1922; Mattick and Wright, 1925]. Exps. 1 and 3, however, show that, where the absorption of calcium was increased by feeding cod-liver oil to animals in the later stages of lactation, the calcium content of the milk was increased. The benefits of increased calcium assimilation would thus be shared by both mother and offspring, since some of the extra calcium absorbed would be available for the young. It is of interest in this connection, as shown by Telfer and Crichton [1924], that even small changes in the mineral composition of goat's milk may affect the nutrition of young kids. In the case of the animals in early lactation (Exps. 2 and 4), there was no such increase in the calcium content of the milk.

Iodine, however, differs from the other mineral constituents of milk in the ease with which its concentration may be increased by feeding. This increase has been shown to occur when iodine is given either in what is presumably organic combination in cod-liver oil, or in inorganic form as potassium iodide. These findings confirm the results of earlier experiments at this Institute [Orr and Leitch, 1927]. Preliminary trials by dialysis and ultra-filtration also showed that, of the total normally occurring in goat's milk, about 80 % was in ionic form and that, when the increase took place after either cod-liver oil or potassium iodide, the relative proportions of ionic and colloidal iodine remained unaffected.

The effects of cod-liver oil on the fat content were much more indefinite. Exp. 1 showed a reduced percentage in the oil-period but with the rise in volume the average daily total secretion of fat remained the same. In the second experiment changes in the fat content were insignificant, while in the third it varied inversely as the volume. In Exp. 4 it was lowest in the oil-period, but in this experiment with the fall in volume in the post-period there was a marked rise in the fat content. These results are not in agreement with those of Drummond *et al.* [1924] and Golding, Soames and Zilva [1926], who concluded that cod-liver oil produced a reduction in the yield of milk-fat which was greater than might be attributed to the natural decline with advance of lactation; they are rather in agreement with those of Taylor and Husband [1922], who stated that, after feeding rations high in each of the constituents, protein, carbohydrate and fat, the percentage composition of milk was determined by its rate of secretion and that diet had no direct influence on it, but affected it only indirectly through changes produced in the daily volume.

The single experiment in which cod-liver oil was replaced by an equal volume of olive oil and an equivalent amount of iodine as potassium iodide did not show that the beneficial effects of cod-liver oil could be reproduced by such a change. Although the addition of small amounts of potassium iodide to a cereal ration fed to growing pigs produced increased balances of nitrogen and phosphorus [Kelly, 1925], it must be emphasised that the amounts of iodine were greatly in excess of those which had previously been given in cod-liver oil by Husband, Godden and Richards [1923]. In this experiment

and in the one in which olive oil was given alone there was an increase in the fat content of the milk in each case in the second half of the experimental period. These results are complicated by the fact that in one case the volume was falling and in the other rising and it does not seem possible to ascribe the effect to either the iodine or the oil. The olive oil experiment, however, shows that the increase in the iodine content in Exp. 5 must have been due to the potassium iodide and not to any effect of the oil *per se*.

Summary.

1. The feeding of cod-liver oil to goats improved the balance of calcium by reducing the amount excreted in the faeces. The effects on phosphorus excretion were variable.

2. In the case of animals in late lactation, the percentage and total amounts of calcium in the milk were increased by feeding cod-liver oil.

3. The amount of iodine in the milk was increased by feeding either cod-liver oil or potassium iodide.

4. The fat content of the milk showed no definite changes as a result of feeding cod-liver oil.

5. In a single experiment in which cod-liver oil was replaced by olive oil plus an equivalent amount of iodine as potassium iodide, the effects of the former were not reproduced.

To Dr J. B. Orr, Director of the Rowett Research Institute, and Dr H. E. Magee, Head of the Physiology Department, my thanks are due for their kind help and criticism. I am also indebted to Miss A. E. Glennie for assistance in the analytical work.

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CLXVIII. THE GLUTATHIONE CONTENT OF CHICKEN SARCOMA: A REJOINDER

By HIDETAKE YAOI and WARO NAKAHARA.

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(*Received September 19th, 1927.*)

The recent paper on "Quantitative studies of the nitroprusside reaction in normal tissues and tumours," by Kennaway and Hieger, appearing in this *Journal* [1927], seems to call for a brief comment.

A year ago we published our observations on the same subject [Yaoi and Nakahara, 1926] and reported that the Rous chicken sarcoma contains but little glutathione. This fact seemed especially interesting because of the abundant occurrence of glutathione in transplantable mammalian tumours, such as rat sarcoma and rat and mouse carcinomas, which are known to be similar to the avian tumour as regards their rate of tissue respiration and glycolysis [Murphy and Hawkins, 1925]. Kennaway and Hieger alleged that our results are incorrect, on the basis of the supposition that we used "glutinous and largely non-cellular material" from the centre of the overgrown tumours, instead of actively growing sarcoma tissue. This allegation is totally unfounded.

Nobody who is at all familiar with experimental cancer research would think of using central necrotic material for any study of tumour tissue. In our experiments we took the actively growing peripheral layer of the tumour, taking care not to include any adherent muscular tissue. The practice is so universally adopted by all experienced workers that we did not feel constrained to mention it in our paper.

In view of the quoted allegation, it is important to compare our figures. We reported three determinations, namely: 12, 13 and 20 mg. glutathione per 100 g. of fresh sarcoma tissue, based on the starch method. Kennaway and Hieger's figures for the same method ran: 9·6, 14, 20, 24, 27 and 54 in the case of intramuscularly implanted tumours, and 18, 21, 21, 24, 29, 30, 41, 47, 51 and 52 in those subcutaneously implanted.

We would call attention to the very great variation of their figures. Even allowing for a considerable experimental error, we can hardly see how such a wide range, as from 9·6 to 54 mg. %, for example, can be obtained by using any uniform material. From the information given it appears that they used only very young tumour grafts (6–11 days old) for their test. It is probable that their higher figures are attributable to the admixture of the reactive

tissue elements which, as is well known, are associated with the young tumour grafts, often in a considerable amount.

We feel obliged to point out another objection. Kennaway and Hieger report two instances where the nitroprusside method gave nil in both cases and the starch method 20 and 21 mg. %, and yet their data include two other cases in which the nitroprusside method gave 12 and 13 mg. %, while the starch method gave only 14 and 18 mg. % respectively. Are we to understand that, with the starch method, materials that gave 0 % with nitroprusside will yield a higher value than other materials that gave 12–13 mg. % with nitroprusside? This is an obvious self-contradiction, which seems to us to require some sort of explanation. In the face of these facts Kennaway and Hieger's figures do not seem reliable.

It seems to us that the difference between the results of Kennaway and Hieger and our own is not only apparent in the case of the Rous sarcoma, but also in the case of normal tissues, especially muscle. Tunnicliffe [1925], whose method we are following, states that "the figures obtained for skeletal muscle have been exceedingly constant," and mentions 33–35 mg. % for rat, and 40–44 mg. % for rabbit muscle. We reported for chicken muscle 44 and 56 mg. %. These are in contradiction to the extremely low figures quoted by Kennaway and Hieger, namely: 16, 18, 19, 21 and 26 mg. %. These figures are so abnormally low and variable for this type of tissue that they lend themselves to the suspicion that they might be due to insufficient extraction. We ourselves have experienced some difficulty in finely grinding up muscle fibres.

Kennaway and Hieger conclude that human as well as animal tumours are not distinguished by any deficiency of glutathione. We are in agreement with this view, and we previously stated that glutathione is present in abundance in the malignant tumours, other than the Rous sarcoma, and in the normal tissues that we examined. However, the chicken sarcoma is exceptional in that its glutathione content is peculiarly small, considering its very active metabolism.

It may not be amiss to add here that our recent experiments show that the active layer of the Rous sarcoma has at best a very feeble reducing action on methylene blue [Yaoi and Nakahara, 1927]. Under the experimental conditions in which skeletal muscle of the chicken completely reduces methylene blue in 16 hours, the sarcoma tissue may fail to produce any perceptible reduction for 72 hours. If the glutathione content of the chicken sarcoma were as great or greater than that of the muscle, as claimed by Kennaway and Hieger, one would scarcely expect this exceedingly feeble reducing action on methylene blue on the part of the chicken sarcoma.

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CLXIX. THE ABSORPTION SPECTRA OF OILS AND OIL CONSTITUENTS WITH SPECIAL REFERENCE TO PRO-VITAMIN D.

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(*Received September 24th, 1927.*)

Ergosterol, which shows three absorption bands at 293·5$\mu\mu$, 281·5$\mu\mu$ and 270$\mu\mu$ respectively undergoes the reaction

$$\text{ergosterol} \xrightarrow{\text{light}} \text{vitamin D.}$$

[Heilbron, Kamm and Morton, 1927; Pohl, 1927; Windaus and Hess, 1927; Rosenheim and Webster, 1927.] Various investigators have shown that vegetable oils also acquire antirachitic potency on irradiation. We have therefore sought to determine whether ergosterol can be detected in vegetable oils by means of the spectroscope.

The possibility of other absorbing constituents in the oils, masking the presence of ergosterol, must first be considered. Aliphatic esters rarely, if ever, exhibit marked selective absorption. The type of curve found corresponds in most cases with feeble general absorption in the near and middle ultra-violet regions, and more powerful end absorption in the extreme ultra-violet. There is no evidence that the glycerides of fatty acids behave differently from the simple esters. Free fatty acids do not exhibit selective absorption to any marked degree. It has been shown [Heilbron, Kamm and Morton, 1927] that pure cholesterol shows no selective absorption, and we have recently found that sitosterol, which is the chief constituent of the unsaponifiable matter of vegetable oils, shares this property.

Hence, the detection of ergosterol in oils by means of the spectroscope will depend only on (*a*) the chance occurrence of substances peculiar to a particular oil, or particular process of extraction, and (*b*) the capacity of small amounts of the selectively absorbing ergosterol to show themselves in the presence of the general absorption of the other constituents already mentioned. This was put to direct test.

A very transparent sample of kapokseed oil, showing no selective absorption in the ultra-violet, was used as a solvent for ergosterol. Thin films of oil of a thickness of the order 1/10 mm. were transparent down to 240$\mu\mu$.

With the addition of 0·2 % of ergosterol, the three bands were detectable with certainty, while lesser amounts gave positive results, although these results were not always unequivocal. The sensitivity of the spectrographic method will depend primarily on the transparency of the oil under investigation, and in many cases of clear samples of high purity, percentages as low as 0·01–0·02 have been detected, *e.g.* cottonseed oil.

In what follows a "negative" test does not, therefore, *exclude* a concentration of ergosterol less than 0·2 %.

The reaction, ergosterol ——(light)—→ vitamin D is accompanied by the appearance of a new band at 247 $\mu\mu$ which is, in all probability, characteristic of the vitamin [Morton, Heilbron and Kamm, 1927]. Unfortunately a very considerable concentration of pure vitamin (of the order of 0·01 g. per 100 cc. alcohol) is necessary to secure the detection of this absorption band. In view of the fact that such a concentration is equivalent to some thousands of doses of cod-liver oil (in respect of vitamin D), it is not surprising that the test will not serve as a criterion of the absence or presence of the antirachitic vitamin. As a further indication of the limits of the spectroscopic method, it may be pointed out that cod-liver oil, the source of our original "active" cholesterol, does not exhibit the pro-vitamin bands, although it does, of course, contain the precursor substance. A pronounced inflection, only, is shown in the same region as the ergosterol bands. It will thus be seen that the method only enables us to discriminate between oils containing relatively little ergosterol, and those containing quite reasonable amounts.

A large number of oils have been subjected to spectrographic examination, and the results are summarised in Table I. (See also Figs. 1—5.)

Certain of these oils will now be discussed in more detail.

Cottonseed oil. The unsaponifiable matter obtained by the saponification of a large quantity of oil, gave a high yield of crystalline sterol, which was fractionally crystallised from ethyl acetate, the various fractions then being examined spectroscopically. The least soluble fraction was found to contain 2·5 %, the most soluble 0·75 % of ergosterol. Cottonseed oil would thus appear to be a very rich source of this compound.

Sesame, illipi nut, shea nut and pumpkinseed oils. These all show well-defined selective absorption in the middle ultra-violet. Since, however, these bands do not exhibit the fine structure characteristic of ergosterol, there is no valid reason for connecting them with the pro-vitamin. Moreover, a preliminary examination of the unsaponifiable matter of shea nut oil has so far given no indication of the presence of ergosterol.

Yeast-fat. As would be expected from the work of Smedley MacLean and Thomas [1920], who found it to contain as much as 40 % of ergosterol, this fat gave by far the most pronounced absorption of all the oils examined.

Ground nut oil. This was probably the most interesting oil examined, showing as it did, big variations from sample to sample. In some cases there was no sign of selective absorption, in others very well-defined ergosterol

bands were obtained. Moreover, in all cases where the ergosterol bands were plainly present, there was also a more or less well-defined band in the neighbourhood of 320$\mu\mu$. This latter band has only been found hitherto in oils of animal origin such as wool-fat, whale oil, cod-liver oil, etc. [cf. Schlutz and Morse, 1925; Heilbron, Kamm and Morton, 1927], and there is no definite evidence of its connection with vitamin chemistry. Experiments are, however, now in progress, which have been designed to elucidate the nature of the compound exhibiting this band.

Table I.

Oil	Ergosterol bands	Band at or near 320 $\mu\mu$	Remarks
Olive	–	–	Band at λ max. = 407 $\mu\mu$
Nux vomica ...	–	–	
Tiger nut	–	–	
Alfalfaseed	Very feeble	–	
Coconut	–	–	
Babassu	–	–	
Yeast-fat	+ + + + +	–	
Sesame	?	–	Well defined band at λ max. = 287 $\mu\mu$
Kapokseed	–	–	
Rape	–	–	
Soya bean	–	–	
Mowraseed	Inflection 265–280 $\mu\mu$	–	
Illipi nut	?	–	Selective absorption λ max. = 270$\mu\mu$
Shea nut	?	–	,, λ max. = 278$\mu\mu$
Cottonseed	+ +	–	
Linseed	+ +	–	
Maize...	+ +	–	
Ground nut sample A	Inflection	–	
,, ,, B	–	–	
,, ,, C	–	–	
,, ,, D	+	–	
,, ,, E	+ +	+	
,, ,, F	+	–	
,, ,, G	+ +	+ +	
,, ,, H	+ +	+ +	
Brazil nut	–	–	
Palm oil	–	–	
Palm kernel ...	–	–	
Rice meal	–	–	
Pumpkinseed ...	?	–	Well defined selective absorption λ max. = 276·5 $\mu\mu$. No fine structure
Sunflower	–	–	
Whale	–	?	
Wool-fat	Inflection	+ +	
Cod-liver oil	?	+	
Ditto. Unsap. ...	?	+	
Extracted C.L.O. ...	?	+ +	
"Ostelin"	?	+	

Olive oil. Lewkowitsch [1927] examined the absorption of olive and tea oils. A band at 275$\mu\mu$ was observed in the former oil. In view of the frequent contamination of olive oil with cottonseed oil, and also since a sample known to be free from adulterants, examined by us, did not show any band, the observation of Lewkowitsch should be accepted with reserve.

The activation of oils by irradiation bears a close relation to the stimulation of certain vital processes in phototherapeutics. It is well known that skin is

rich in cholesterol and that there is reason to suppose that a part at least of the mechanism of light therapy is the formation of vitamin D in the epidermis. This has not yet been proved to be true.

Thin sections of skin have been obtained by us (by the kindness of Prof. Blair Bell) and examined spectroscopically. The results were in approximate agreement with the data of Glitscher and Hagenbach [1926]. Sections of 0·1 mm. were not transparent below 297 $\mu\mu$, and with thinner sections it was difficult to retain continuity when the sections were placed between quartz plates. Several experiments with very thin sections were carried out, and although inflections were shown in the middle ultra-violet regions of the curves, more definite evidence of selective absorption could not be obtained. Such a result is not surprising as the concentration of ergosterol in the epidermis must in any case be exceedingly small.

Summary.

1. A method of detecting and estimating ergosterol in oils by direct spectrographic examination has been studied and its limits have been defined.

2. A large number of oils and oil extracts have been examined, and their absorption curves have been obtained.

In conclusion we desire to express our thanks to Messrs J. Bibby and Sons, Ltd., to Messrs Joseph Nathan & Co., Ltd. (proprietors of Glaxo), and to Messrs R. Silcock and Sons, Ltd., for samples of oils, and to the Food Investigation Board of the Department of Scientific and Industrial Research for a grant which has enabled the work to be carried out.

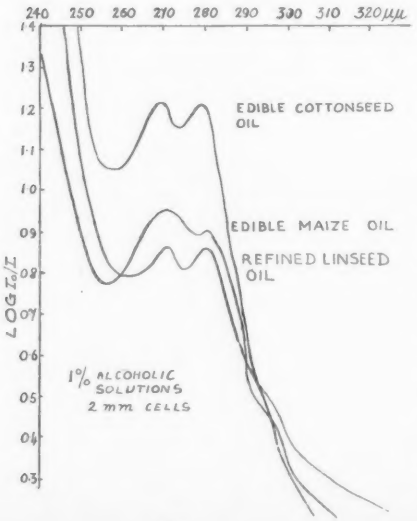

Fig. 1

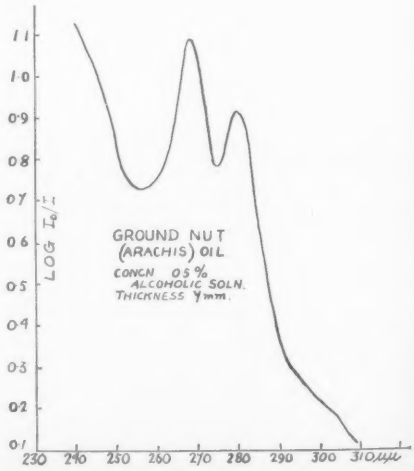

Fig. 2.

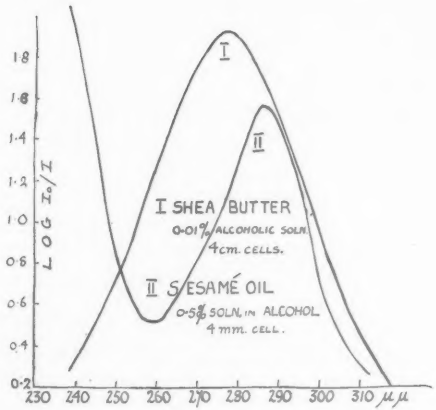

Fig. 3.

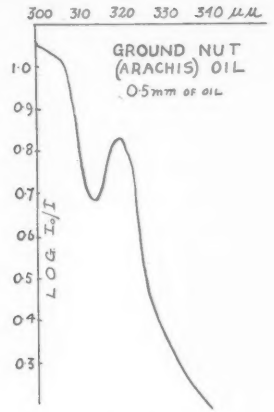

Fig. 4.

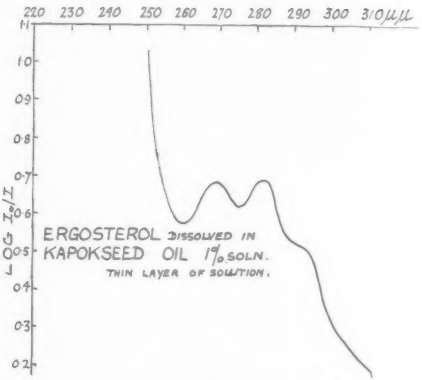

Fig. 5.

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CLXX. INVESTIGATIONS ON GELATIN.

PART IX. THE SCISSION OF GELATIN INTO CONSTITUENT PROTEINS.

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Recent investigations carried out in this laboratory indicate that gelatin consists of molecules, or aggregates of molecules, which readily undergo intramolecular change. It has been shown, for example, by Knaggs [1923] that if gelatin is hydrolysed rapidly by throwing it into boiling 25 % hydrochloric acid solution, it yields a distinctly smaller fraction of basic products precipitable by phosphotungstic acid than it yields when it has been allowed to stand with the cold acid solution before boiling. This phenomenon has been further investigated by Schryver and Buston [1927], who have found that the increased amount of basic product obtained by treating the gelatin with cold acid before hydrolysis is due mainly to the presence of *dl*-lysine, which cannot be ascribed merely to the racemisation of the *l*-lysine, since the latter occurs along with the optically inactive base in normal (or slightly more than normal) quantities. Thornley [1927] has found that the treatment of gelatin with relatively dilute cold solutions of acids or alkalis can give rise to changes in the amounts of basic products obtained when the sample thus treated is afterwards submitted to rapid hydrolysis with strong acids. Furthermore, Knaggs and Schryver [1924, 1] have shown that when gelatin is heated in water and then hydrolysed by boiling acids in the usual way, there is in the hydrolysis products more nitrogen in the form of non-amino-nitrogen than in those obtained in a similar way from gelatin not previously heated with water—indicating that this comparatively mild treatment causes the formation of stable nitrogenous rings, which are not opened up by subsequent hydrolysis with strong acids.

Electrolysis of gelatin hydrochloride and sodium gelatinate.

In view of the accumulating evidence of the readiness with which structural change takes place within the molecules, it was of interest to ascertain whether gelatin possesses the same structure when functioning as anion and as cation.

Some evidence has been afforded by the investigation of ultra-violet absorption spectra by Baker and Davidson [1925] that two distinct forms of gelatin exist. The experiments recorded in this communication tend to confirm the conclusions drawn by these authors.

For the purposes of the experiments, a gelatin was employed which had been freed from ash and separated from simpler degradation products, which are present in ordinary commercial samples, by flocculation in an electric field [Knaggs, Manning and Schryver, 1923; Knaggs and Schryver, 1924, 2]. The flocculum thus obtained was thoroughly washed on a filter with cold water. Two portions of this were taken and dissolved in water; to one solution hydrochloric acid was added to bring it to p_H 3·0, and to the other sodium hydroxide to bring it to p_H 8·0 or 9·0. The gelatin was precipitated from these solutions by electrolysis, the poles being separated from the gelatin solutions by means of semi-permeable membranes[1].

When the gelatin was deposited cationically by the electrolysis of the hydrochloride, the lower pole of the apparatus was made the cathode; when, on the other hand, it was deposited anionically by electrolysis of sodium gelatinate, the lower pole was the anode. The upper poles were washed with a continuous stream of CO_2-free water. Electrolysis was carried out with very small currents, which hardly ever exceeded 10 milliamps., and were generally appreciably less. Noticeable heating effects were thereby avoided. The electrolysis was continued until the washings of the upper pole showed no traces of chlorine, or of alkali. At this stage, with a potential fall of 20–30 volts per centimetre, the current passing had usually fallen below 1 milliampere.

The difference between the appearance of the gelatin deposited cationically and that deposited anionically was extremely well marked. The former separated as a compact, almost transparent gel on the collodion membrane, from which it could be peeled off as a single solid mass by a knife. The gel occupied only a relatively small bulk of the whole system, and the supernatant liquid was water-white and clear; it contained only a small amount of nitrogen, which amounted to about 3·5 % of the total originally present in the solution. The gelatin deposited anionically formed, on the other hand, a bulky flocculum extending from one half to two-thirds of the way up the cylinder, while the clear liquid in which this flocculum was suspended was relatively rich in nitrogen, containing nearly 18 % of the total. (See Experimental Details, Series A.)

By the electrolysis of sodium gelatinate, therefore, a considerable portion of the nitrogen remains unprecipitated, and two distinct fractions are thus obtained, *viz.* a soluble and an insoluble.

[1] The apparatus employed was similar to that figured in the *Biochem. J.* 1924, **18**, 1082. Simple cylinders were used instead of bell-jars, and a number of such units were placed either in series or in parallel, so that several experiments could be carried out at one time. By a suitable arrangement of plugs and switches the current passing through any unit could be measured when required. Platinum electrodes were employed in all cases.

The insoluble ana-fraction.

The insoluble fraction, provisionally designated insoluble ana-gelatin, carries down with it a certain amount of the soluble fraction—soluble ana-gelatin—which at the conclusion of the electrolysis is not distributed evenly throughout the liquid, but tends to pass with the insoluble fraction towards the anode. Most of the soluble portion is, however, readily removed from the insoluble ana-gelatin by washing. If the latter be shaken thoroughly with ice-cold water, and the mixture be then centrifuged, a certain amount of nitrogen will be found in the supernatant liquid. If these processes of washing and centrifuging be repeated several times, it will be found that the nitrogen in the supernatant fluid rapidly diminishes with successive treatments until after three or four such treatments it becomes approximately constant, amounting to about 3 mg. per 100 cc. of the liquid. This must be taken as a measure of the solubility of this fraction in water, and it indicates that this so-called insoluble fraction is soluble in water to the extent of about 15 parts in 100,000. (See Experimental Details, Series B.)

The soluble portion removed by these washings may not represent the total amount of gelatin in the soluble form, since a part of this remains attached to the insoluble, and can only be separated from it by repeated flocculations from neutral or alkaline solution. Probably it is enclosed within the particles, from which it is only set free by repeated complete dispersions in water.

If the insoluble fraction, freed as above from the soluble product, be dissolved in water, and flocculated in an electric field, it separates out readily as a bulky flocculum. The nitrogen remaining in the supernatant fluid, however, even after repeated flocculations, is appreciably higher than that due to the actual solubility of this fraction when freed from the soluble ana-gelatin (*i.e.* about 3 mg. nitrogen per 100 cc.). (See Experimental Details, Series C.) The insoluble product was also converted into the sodium salt and into the hydrochloride, and separated again from the solution of these salts by means of electrolysis. In both cases it was precipitated in the form of a flocculum. When separated from the solution of the hydrochloride, the nitrogen remaining in the supernatant liquid was lower—when separated from the sodium salt, higher—than the nitrogen remaining in the supernatant fluid when the unionised product was flocculated. In all cases, however, it was higher than 3 mg. per 100 cc. (See Experimental Details, Series A, Part 2.)

These facts seem to the authors to have a definite significance. In an earlier paper of this series [Knaggs, Manning and Schryver, 1923] it was found that gelatin after being freed from inorganic salts readily separated from dilute solution as a flocculum. After repeated separation from solution ("recrystallisation") the nitrogen in the supernatant fluid became constant, and it was assumed at the time that a product had been obtained which was in equilibrium with its saturated solution. Certain facts, however, militated against this

assumption. It was found that, if a solution were prepared containing enough gelatin for saturation and this were concentrated at low temperatures to a small bulk, the gelatin did not always separate, as it would have been expected to do from a supersaturated solution. This was especially the case if the saturated solution had been allowed to stand a day or two before concentration.

These facts indicate that "insoluble" gelatin cannot be dispersed in water at 25–30° without undergoing a certain amount of change and of conversion to the soluble form, and Manning [1924] showed that the most highly purified gelatin obtained up to the time of his work underwent a distinct irreversible change at a slightly higher temperature (37°) which was readily revealed by viscosity measurements. In the experiments described in detail later (Series A, Part 2 and Series C) it would appear that, on dispersion in water at 30°, from 3–5 % of the insoluble ana-gelatin is converted into a soluble non-flocculable form.

The soluble ana-fraction.

This was obtained by concentration of the supernatant fluid and washings from the anionic flocculation at room temperature. No gel was obtained, but a thick syrupy solution, from which the protein could be obtained by means of acetone as a white granular powder. (See Experimental Details, Series D.) This is a substance of high molecular weight, 50,000–60,000, which appears to vary slightly with the concentration [cf. Horne, 1924]. (See Experimental Details, Series E.)

The protein is readily precipitated from the syrup by saturated ammonium sulphate. Precipitation commences at 28·5 % saturation, and is complete at or about 50 % saturation. Its solution normally possesses a p_H of 4·8, which is probably its isoelectric point, whereas the p_H of the suspension of the insoluble product was 5·8. The latter could be dried in the form of sheets, not distinguishable at first sight from those of ordinary gelatin. (See Experimental Details, Series F.)

Attempt to resynthesise a gel from soluble and insoluble ana-gelatins.

It was thought that it might be possible to obtain a gel by recombination of the soluble and insoluble ana-gelatins, since neither fraction alone was in the form of a gel. These were accordingly mixed in the proportions in which they were obtained by the electrolysis of their sodium salts as above, and the solution of the mixture was dried down into a sheet at room temperature. The sheet was redissolved in water, and to the solution hydrochloric acid was added to bring it to p_H 3·0. The gelatin was then separated cationically by electrolysis. It did not, however, come down in the form of a gel, but as a flocculum. The supernatant fluid, furthermore, contained the same amount of nitrogen as would have been found in that from (*a*) the electrolysis of the hydrochloride of the insoluble fraction alone, plus (*b*) the nitrogen of the added amount of soluble fraction. There is no evidence, therefore, that the soluble

and insoluble ana-gelatins can be made to recombine to form a gel. Now certain other facts have been discovered, which offer an explanation of this negative result.

It was found that the cata-gelatin gel, obtained by the electrolysis of the hydrochloride, gave on hydrolysis a diamino-nitrogen value of about 29. The corresponding number for the insoluble ana-gelatin was 23·5, while that for the soluble ana-gelatin was found to be 19. These numbers indicate, therefore, that the molecules in the ana- and cata-gelatins possess a different structural arrangement, and thus the cata-gelatin gel is not a simple combination of the soluble and insoluble ana-fractions. (See Experimental Details, Series H.)

The relationship of the separated proteins to one another, and the mechanism of gel formation.

Proteins yield on hydrolysis in many cases single amino-acids in very small amounts. If these are formed from a single kind of molecule, or from aggregates of the same kind of molecules, the latter must be of very large size. It is conceivable, however, that proteins may be made up of aggregates of different kinds of molecules, each with a skeletal polypeptide structure, held together in some form of loose combination. Many attempts, which need not be discussed here in detail, were made to separate pure gelatin into such constituents, by its submission, after various treatments, to the action of an electric field. It is possible that by the electrolysis of the sodium salts as described the scission of gelatin has been effected into constituent proteins (which may have undergone intramolecular change during the process) which have no known relationship to one another, beyond their association together to form the gelatin complex.

There is, however, another possible explanation of the results, which needs discussion, according to which the two proteins obtained by the electrolysis of sodium gelatinate are much more intimately related to one another. Before dealing with this possibility, however, it is necessary to digress for a moment in order to discuss the effect of heating gelatin with water.

When gelatin is warmed with water, it undergoes an irreversible change, and some experiments, quoted above, indicate that this takes place to a small extent when a flocculated gelatin is dissolved, or rather dispersed, in water. Hitherto, it has been generally assumed that this change is of a degradative nature, accompanied by the scission of some of the polypeptide linkages. Some colour is given to this assumption by experiments recorded by various authors, and by the fact that commercial preparations of gelatin contain simple nitrogenous constituents which readily pass through the membranes used for dialysis. A change into simpler nitrogenous products readily takes place when the gelatin samples have not been completely freed from salts, and also occurs during its preparation on an industrial scale from its precursors, which, before the extraction process, have been submitted to the action of

fairly strong acids or alkalis, which are not completely removed by subsequent washing with water. (See Experimental Details, Series A, Part 2.)

Now experiments carried out in this laboratory on an ash-free gelatin, which had been separated from simple nitrogenous products by flocculation in an electric field followed by thorough washing of the flocculum, indicate that the irreversible change produced by heating with water is not of the degradative character mentioned. The authors are indebted to Dr A. B. P. Page for these experiments. (See Experimental Details, Series K.)

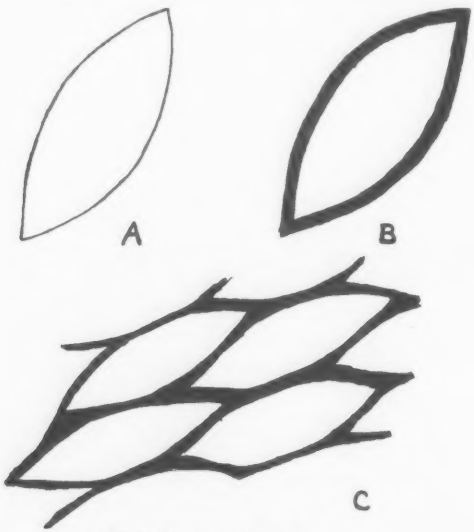
It was found that in the case of a 2 % solution of a purified gelatin which had been boiled for 22 hours under a condenser—a treatment which produces a profound change in its properties—no evidence of peptide scission could be detected, either by the Foreman method of titration in alcohol, or by Van Slyke's nitrous acid method. When, however, Sørensen's formaldehyde method was employed, and the titration was carried out electrometrically, it was found that the heated gelatin solution required a very small amount more alkali to bring the E.M.F. to 650 millivolts than did the unheated portion. If this slight excess required be ascribed to the scission of peptide linkages, then the nitrogen involved in this process amounts to distinctly less than 1 % of the total, and it is extremely improbable that this just detectable change can account for the marked changes in the properties (such as solubility, flocculability, etc.) of the solution.

A similar conclusion was arrived at by an entirely different series of experiments. A gelatin solution which had been boiled for 5 hours (during which period the greater part of the viscosity change had taken place) was submitted to fractional dialysis. This was carried out in hollowed ebonite cylinders, which were clamped together at their flattened edges, between which was secured a collodion membrane, which thus separated the two cylinders. Into one cylinder was introduced the heated gelatin solution, and into the other water. Arrangements were made to secure a difference between the pressures on the two sides of the membrane, to accelerate the rate of dialysis and prevent dilution of the gelatin solution. A membrane of small permeability, softened by the method of Brown [1922] in 60 % alcohol, was first used, and dialysis was continued until no further nitrogen could be detected in the dialysate. The membrane was then replaced by a more permeable one (soaked in 80 % alcohol). After completion of a second dialysis, the heated gelatin solution was submitted to a third, using a membrane soaked in 90 % alcohol, the most permeable collodion membrane which can be manipulated without difficulty. As a result it was found that only minute traces of nitrogen passed the membranes, even when the most permeable one was employed. It appears, therefore, as a result of these experiments, that the irreversible change in gelatin produced by the action of water is not due to peptide scission, but to the change into a soluble, non-gelling, complex protein.

In the literature the term "meta-protein" has been suggested for natural

proteins which have undergone some change during "denaturation," such as coagulated egg-albumin. This term may be conveniently retained to include products which have been obtained from proteins without measurable peptide scission. The products obtained by heating with water a purified gelatin may therefore be designated "meta-gelatins."

To revert now to the discussion of the explanation of the relationship between the two protein fractions, soluble and insoluble, of gelatin. Evidence has been given which indicates that a certain amount of meta-gelatin is produced when a flocculated gelatin is dispersed in water at 30°. If one assumes the micellar structure for gelatin, then it is conceivable that during the dispersal process, the peripheral part of the micellar structure undergoes change into the soluble meta-gelatin. When the changed micellae are sufficiently close together, the outer layers of meta-gelatin could form a continuous phase and thus produce a gel, as shown in the diagram.



A. Micella in original flocculum.
B. Micella surrounded by meta-gelatin.
C. Gel.

On cooling dilute solutions, however, the unchanged gelatin would separate as a flocculum, whereas the greater part of the meta-gelatin would remain dissolved in the water-phase, a small amount being also carried down with the flocculum. If this view is correct, a more complete separation of the gelatin and meta-gelatin takes place after electrolysis of the sodium salts than after electrolysis of the acid or neutral solution. The products provisionally called insoluble and soluble ana-gelatin would therefore be simply gelatin and meta-gelatin, which have undergone intramolecular change during the electrolysis

of the solution of their sodium salts[1]. In the cata-form, the soluble and insoluble fractions appear to be in some form of combination, possibly due to the presence of the free amino-group of *dl*-lysine in the insoluble fraction (*vide supra*, p. 1284) and the somewhat acidic character of the soluble meta-gelatin.

The data at the moment available are not sufficient for determining which of the above explanations of the relationship of soluble ana-gelatin to insoluble ana-gelatin is correct. By the employment of the experimental methods described above, it may, however, be possible to arrive at a definite solution of the problem. By heating gelatin with water for a relatively short time, it is possible to convert a small part of the gelatin into meta-gelatin. The two fractions could then be separated either by flocculation in an electric field or by electrolysis of their salts. The floccula or gels thus obtained could then be heated again and a further fraction of soluble product obtained. This process could be repeated until no more insoluble fraction remains. By systematic investigation of the soluble fractions it should be possible to ascertain whether gelatin is made up of several different proteins, or whether the insoluble gelatin consists mainly of one class of molecules, which by the action of hot water are converted by stages into the soluble form. Work on these lines is now proceeding.

The authors, before concluding, would like again to draw attention to the importance of considering the intramolecular changes which take place so readily when gelatin is submitted to mild treatment. They are of the opinion that the ordinary physico-chemical methods, the results of which have been interpreted on the assumption that gelatin is simply a colloidal ampholyte, are not, by themselves, sufficient to explain the various properties, including gel formation, of this product.

Experimental details.

Series A. Part 1.

Cationic flocculation. 3·5 g. of gelatin, freed from ash by washing and electrodialysis and freshly flocculated from 1·5 % neutral solution, were washed

[1] The view that gel formation is due to the presence of soluble and insoluble products is, of course, not new, though the above method of presentation probably is. The view has recently been subjected to experimental investigation by Northrop and Kunitz [1926]. They claim to have partially separated gelatin, purified by Loeb's method, into fractions differing in their solubilities in water. The authors of this paper are of opinion that the methods adopted by Northrop and Kunitz are open to criticism on various grounds. In the first place, their own experiments indicate that a gelatin, purified by Loeb's method, still contains sufficient degradation products to account for almost the whole of their soluble fraction. (See also Experimental Details, Series A, Part 2.) Secondly, the repeated solution of their precipitates of the more insoluble fraction in water at 32° must result in the partial conversion of the more insoluble fraction of the gelatin into the more soluble. It is probable that the two fractions obtained by these workers represent two different mixtures of soluble and insoluble gelatin, more or less contaminated with degradation products. A much more convenient method of separating gelatin into soluble and insoluble fractions has been described in previous papers of this series, which have been completely ignored by Northrop and Kunitz.

on the filter and made up to 1·5 % solution by warming at 40°. The solution was brought to p_H 3·0 with HCl and submitted to a potential difference of 200 volts in the apparatus previously described. After 6 days no further acid could be detected in the anode liquor and the current passing was 0·2 milliamp. The nitrogen in the solution was found to vary from a very low value at the top to 4·4 mg. per 100 cc. towards the bottom of the vessel. The bulk of the gelatin was precipitated as a hard clear gel on the lower membrane, about 1" thick. This cake was broken up and the solid filtered off.

N in supernatant liquid (200 cc.)	6·8 mg.	Total soluble
N in filtrate from broken solid (30 cc.) ...	6·3 mg.	N = 13·1 mg.
N in original solution	380·0 mg.	
Hence N not precipitated	3·5 % of total N	

Anionic flocculation. A similarly prepared solution was brought to p_H 8·0 with KOH, by titration. An apparatus completely enclosed was used in order to avoid absorption of CO_2 from the air by the alkaline solution, freshly boiled distilled water being employed throughout for the same purpose. After 24 hours under a P.D. of 200 volts a bulky white flocculum was precipitated, and after 4 days no further alkali could be detected in the cathode liquor. The flocculum was filtered off and the nitrogen determined in the solution.

N in supernatant liquid (125 cc.) ...	29·0 mg.	Total soluble
N in filtrate from flocculum (100 cc.)	25·6 mg.	N = 54·6 mg.
N in original solution	310·5 mg.	
Hence N not precipitated	17·7 % of total N	

Thus from the acid solution almost the whole of the gelatin is obtained as a clear gel, while from the alkaline solution a considerable portion of the nitrogen remains unprecipitated and the rest is obtained as a flocculum.

Series A. Part 2.

Flocculations of crude gelatin. A series of flocculations was carried out on samples of unpurified gelatin with a view to determining whether (*a*) separation into two fractions by anionic flocculation, and (*b*) precipitation as gel by cationic flocculation took place. The proportion of nitrogen of true soluble protein to the nitrogen merely due to soluble breakdown products was also investigated.

A sample of gelatin which had been freed from ash by washing and electrodialysis, but not by flocculation, was separated by electrolysis from acid (p_H 3), alkaline (p_H 9), and neutral solutions; the results after 5 days are given in Table I.

Table I. *Unflocculated ash-free gelatin.*

p_H of original solution (colorimetric measurement)	Type of precipitate obtained	Total N in solution mg. per 100 cc.
5·0	Bulky flocculum	36·4
3·0	Gel + slight flocculum	33·6
9·0	Flocculum (less bulky)	39·7

Thus, using the crude material, a gel may be obtained by means of cationic flocculation, but the soluble nitrogen is high in all cases. Further experiments show that this is due to the presence of diffusible degradation products.

Some Coignet's "Gold Label" gelatin was flocculated directly from a 1·5 % solution at p_H 3·4, the outside of both membranes being washed very completely with a stream of freshly boiled water. After 4 days a clear gel was precipitated and after 9 days the current had fallen nearly to zero. The thick layer of approximately 10 % gelatin gel was removed and the N determined in the supernatant and wash-liquids.

Table II. *Coignet's "Gold Label" gelatin.*

	mg.	% of total N
Total N in supernatant liquid ...	44·7	7·15
Total N in outer liquid at cathode	9·4	1·50
Total N in outer liquid at anode	19·3	3·09
Hence total soluble N	73·4	11·74
And total diffusible soluble N ...	—	4·59

Thus, in the case of crude gelatin, the true soluble ana-gelatin is accompanied by an amount of diffusible degradation products, the proportion of which approximates to 4·6 % [cf. Northrop and Kunitz, 1926]. These substances remain in solution equally when the gelatin is precipitated anionically or cationically, the slight differences observed above being due to the soluble ana-gelatin itself. After flocculation, however, the bulk of the nitrogen remaining in solution is due to the soluble fraction, the diffusible products representing only 0·5 % of the total. Further flocculations reduce this to zero.

The gel obtained from flocculation of the crude gelatin was reflocculated from acid and alkali; the results (Table III), which are typical, show the reduction in the diffusible nitrogen effected by the second flocculation, and also the difference between the quantities of soluble nitrogen in the case of anionic and cationic flocculations.

Table III.

	Reflocculation of gelatin from alkaline solution			Reflocculation of gelatin from acid solution		
	mg. N per 100 cc.	Total N mg.	N %	mg. N per 100 cc.	Total N mg.	N %
N in supernatant liquid and filtrate	18·7	44·88	7·51	11·2	25·76	4·48
N in outer liquid at anode ...	—	0·95	0·38	—	1·60	0·64
N in outer liquid at cathode ...	—	1·08	0·43	—	Trace	—
Total diffusible N	—	2·03	0·81	—	1·60	0·64

Further flocculations of purified gelatin. Similar results were obtained from the large-scale experiments in which the soluble ana-gelatin was prepared. The freshly washed flocculum, corresponding to 36 g. of gelatin, was dissolved at 37°, and made up to 2½ litres. The solution was brought to p_H 9·0, and then electrolysed for 5 days, the current eventually falling to about 1 milliampere. Both poles were kept washed and the liquids afterwards acidified and evaporated down.

Table IV.

	mg. N	% of total N
Total N (approx.)	6000·0	—
N passing anode membrane	19·4	0·36
N passing cathode membrane	9·7	0·16
N in supernatant liquid ...	799·3	13·32

A part of the 0·52 % diffusible nitrogen obtained here and in other experiments on purified gelatins is probably due to the membranes, which are usually found to be slightly permeable at the commencement of an experiment. Lower values are obtained with older membranes.

An additional experiment may be quoted here to illustrate the effect of the p_H on the result of the flocculation. The gelatin used had been freed partially from the soluble fraction by flocculating twice from neutral 1·5 % solution. It was thoroughly washed on the filter, pressed and reflocculated from neutral, acid, and alkaline solutions by redissolving as quickly as possible at about 30° and instantly electrolysing. The nitrogen in the total liquid was determined after 5 days, the precipitate being centrifuged off in the usual way.

Table V.

Original p_H of solution	Total N mg. per 100 cc.	Type of precipitate	N in solution mg. per 100 cc.
5·0	127·2	Bulky flocculum	9·8
3·0	140·0	Clear weak gel	7·4
9·0	146·1	Flocculum (less bulky)	11·3

Thus cationic precipitation seems to cause the two separate fractions to remain held together, whereas in alkaline solutions they tend to separate. Neutral solutions occupy an intermediate position. If now from the values for nitrogen in solution, given above, be subtracted an amount representing the actual solubility of the insoluble fraction the determination of which is given below (Series B), 4·4 mg. per 100 cc., or 3·5 % of the total nitrogen, is found to be the smallest percentage of nitrogen converted to the soluble form by the act of dispersing in water and warming. The neutral and alkaline solutions yield values of 4·9 and 5·7 % respectively.

Series B.

Solubility of the "insoluble" fraction. The residual flocculum from the anionic flocculation described under Series D, representing 87 % of the gelatin, was pressed on the filter and washed thoroughly to be as free as possible from the soluble ana-gelatin.

10 g. of the flocculum (7·5 % gelatin) were shaken for 45 minutes with 10 times the volume (100 cc.) of ice-cold freshly boiled distilled water. The whole mass was centrifuged and the slightly opalescent liquid filtered and the nitrogen determined therein. The residual substance, still containing small lumps unbroken by the shaking, was again shaken with 10 volumes of water and centrifuged, and this process was repeated until an approximately constant value for the nitrogen extracted on each shaking was reached.

Table VI. *Soluble N extracted by shaking with cold water.*

First	shaking	23·8 mg. N per 100 cc.
Second	,,	14·0 ,,
Third	,,	3·6 ,,
Fourth	,,	2·9 ,,
Fifth	,,	3·4 ,,
Sixth	,,	2·5 ,,

Apart from slight irregularities due to the difficulty of completely removing the finely suspended solid from the clear solution, these figures show an approximately constant solubility, after three extractions with water, of 2·5–3·0 mg. N per 100 cc., corresponding to 15 parts of gelatin in 100,000.

Series C.

Further flocculation of the insoluble fraction (*ana-gelatin*). The previous determination of the solubility of gelatin [Knaggs, Manning and Schryver, 1923; Manning, 1924] was based on the nitrogen remaining in solution in the supernatant fluid after repeated flocculations. This figure, 48 parts per 100,000, cannot be regarded as accurate, since the work recorded in the previous section shows that the bulk of the soluble nitrogen is in the liquid immediately surrounding the flocculum, and not diffused equally throughout the supernatant fluid. The following determinations were therefore made on the whole liquid.

The insoluble residue after six extractions with water was warmed to 40° for 2 minutes in 30 cc. water, and cooled instantly. The opaque liquid was at once flocculated. Within 30 minutes a precipitate had formed, and after 2 days' electrolysis the heavy white flocculum was centrifuged off and the nitrogen determined in the filtered liquid.

Nitrogen in solution after flocculation 13·2 mg. per 100 cc.

By repeated flocculation, however, the soluble nitrogen can be reduced to a lower limit than the above. Thus 11 g. flocculum were made up to 33 cc. in water at 37° and flocculated repeatedly. A more and more bulky flocculum was obtained each time, and eventually a constant value for the soluble nitrogen was obtained.

Table VII.

		Time of flocculation (hours)	Soluble N mg. per 100 cc.
First	flocculation	48	26·3
Second	,,	96	11·2
Third	,,	48	12·2
Fourth	,,	96	8·8
Fifth	,,	48	8·1

Longer periods of flocculation lead to the attainment of the equilibrium in fewer operations.

Table VIII.

		Time of flocculation	Soluble N mg. per 100 cc.
First	flocculation	48 hours	22·6
Second	,,	7 days	14·4
Third	,,	5 ,,	6·8
Fourth	,,	3 ,,	6·8

These numbers are only about one-half that for the nitrogen left in solution after flocculating the material which had been thoroughly extracted with water, and this fact seems to indicate that a part of the soluble fraction is enclosed within the micellae, is not removed by extraction with water and can only be set free on dispersion.

This smallest number, 6·8 mg. per 100 cc., is greater than that obtained by the simple extraction with water of the washed flocculum. If the latter number (2·5 mg. per 100 cc.) be subtracted from the former, a figure should be obtained which would give a measure of the amount of insoluble gelatin converted to the soluble form (*vide supra*, p. 1287) on dispersing in water at 37°, namely, 4·3 mg. per 100 cc., or 3·7 % of the total nitrogen. Reference may be made to experiments at the end of Series A, Part 2, which confirm approximately this calculation, the value there given being determined for 30°, and therefore slightly lower than the above.

The very slight irregularities in the final numbers may be ascribed to:

(*a*) different times of flocculation;

(*b*) variation in the resistance of the membranes and of the whole system, and therefore of the current density applied;

(*c*) temperature effects, the outside temperature varying over a range of 5°.

Series D.

Isolation of the soluble fraction. 45 g. of purified gelatin, freed from ash, were flocculated from water once, and the white flocculum filtered and washed with water. Three litres of 1·3 % solution were then made up by warming and brought to p_H 9·0 with KOH, 0·4047 g. being required. This solution was then flocculated, the apparatus being enclosed and the cathode being kept washed to remove alkali. The voltage was slowly raised from 50 to 200 volts, as the resistance of the solution increased, an average current of about 4·5 milliamperes being passed. After 10 days no further alkali could be detected in the cathode washings; the flocculation was therefore stopped, the clear supernatant liquid removed as completely as possible, and the flocculum filtered on paper and washed. The supernatant liquid and filtrate combined showed a nitrogen content of 16·1 mg. per 100 cc., or a total of 480 mg. of soluble nitrogen. Washings from the precipitate contained a further 17 mg. nitrogen. The bulky precipitate was pressed on a Büchner funnel to remove as much as possible of the soluble fraction adhering, and the filtrate liquids, totalling 2·9 litres, were then evaporated to 60 cc. at ordinary temperature, by means of a fan. The resulting clear syrup was poured slowly into 8 volumes (500 cc.) of absolute acetone, stirred thoroughly, and after standing overnight, stirred, filtered, stirred up with a further small quantity of acetone and again filtered. This process was repeated three times. Finally, the precipitate was sucked dry, transferred to a porous tile and dried *in vacuo* for several days.

A dull white granular powder was thus obtained; it was only very slightly

hygroscopic when thoroughly dried, and dissolved in water to a clear pale yellow solution, without warming. It charred on heating, without melting. It was completely precipitated by ammonium sulphate solution, the precipitation limits being between 28·5 and 50 % saturation.

Series E.

Osmotic pressure determinations. Sac membranes of necolloidine were prepared, and soaked in 60 % aqueous alcohol before use [Brown, 1922]. A rapid rise was observed, reaching a maximum in 1–3 days, and afterwards a slow fall. If started above the constant value, a rapid fall was observed, reaching the constant value and remaining there for some days, followed by a slight further fall.

Table IX.

N in final solution mg. per 100 cc.	Constant osmotic pressure (mm.)	Temperature ° C.	Molecular weight
223·2	64·0	20·5	53,300
287·2	76·0	18·5	57,400
302·4	74·5	17·0	61,600

This molecular weight, although varying slightly with concentration [cf. Horne, 1924], is definitely that of a complex protein.

Series F.

Hydrogen ion concentration determinations. In general, solutions of soluble ana-gelatin are more acidic than those of the insoluble portion. The p_H of the syrup from the anionic flocculation was found to be 4·1 and 4·2 respectively, in two preparations. These may have been too acid owing to traces of CO_2 in solution. A quantity of the syrup, diluted with three-quarters of its volume of boiled water, was therefore electrolysed for 3 days till all traces of electrolyte were removed; the resultant solution had a p_H of 4·8. This probably represents the isoelectric point of the soluble ana-gelatin.

The electrometric buffer-titration curve is fairly smooth. It shows a slight point of inflection at p_H 4·8, but no obvious inflection at p_H 7·7 [cf. Wilson and Kern, 1922, 1923].

The p_H of solutions of insoluble ana-gelatin varied from 5·6 to 6·8, and of the flocculum itself from 5·1 to 6·5. A quantity of the substance, freshly flocculated from alkaline solution, was mixed with water without dissolving and electrolysed for 3 days till all traces of electrolyte were removed. The p_H of the precipitate, determined colorimetrically by spreading out on a watch-glass with the indicator, was then 5·8, and of the supernatant liquid 6·1. Probably 5·8 represents the fairest value of the isoelectric point of insoluble ana-gelatin.

It should be noted, parenthetically, that the p_H of the solution after electrolysis of a crude gelatin corresponds with that of the soluble fraction, which is present in large amount, while the p_H of the solution after electrolysis of a purified sample approximates to 5·8, the isoelectric point of the insoluble fraction.

Series G.

Attempt to resynthesise a gel from soluble and insoluble ana-gelatins. To a quantity of the insoluble ana-gelatin some of the syrupy solution of the soluble fraction was added, in such proportion that the added nitrogen was equivalent to 12 % of the total nitrogen in the mixture, this corresponding to the proportion of the soluble ana-gelatin present after anionic separation. The mixture was melted at 37°, and on cooling a fairly clear gel was produced, which was cut up and dried down in the cold, redissolved, made up to p_H 3·0, and flocculated. After 6 days the current had fallen to 0·5 milliampere and the p_H of the anode liquid was 4·5. The compact flocculum obtained was centrifuged off. The nitrogen in solution showed no recombination.

Table X.

Soluble N calculated	mg. per 100 cc.
Due to added soluble ana-gelatin	13·5
Due to insoluble ana-gelatin, corresponding to second flocculation (see Series C)	14·4
Total	27·9
Soluble N found	30·4

The agreement is sufficiently close to leave no doubt that the two fractions, once separated, do not recombine to form a gel.

Series H.

Hausmann numbers of the fractions.

Soluble ana-gelatin. The nitrogen distribution was determined by the micro-method previously described [Thimann, 1926] both on the concentrated syrup before precipitation and on the dried and powdered soluble substance. Close agreement was observed, as the following numbers show, so that the precipitation by means of acetone does not appreciably affect the product. Furthermore, standing with acid in the cold before hydrolysis causes no increase in the basic nitrogen [cf. Knaggs, 1923]. The third set of numbers in the table was obtained after allowing the powder to stand for 24 hours in 18 % HCl.

Table XI.

	Amide-N	Humin-N	Diamino-N	Mono- + non-amino-N	Total N
Syrup	2·8	0	19·2	77·3	99·4
Powder	3·3	Trace	19·3	77·5	100·1
Powder (stood in acid)	3·3	0	18·3	78·4	100·1

In each case these represent the mean of at least two determinations.

Insoluble gelatins. The original flocculum from the anionic flocculation contains still some soluble fraction, as was shown above, but this amounts only to a small percentage of the total, and hence should not greatly affect the hydrolysis numbers. A slight increase in the amide-nitrogen of the unwashed gelatin may be due to the soluble fraction present. The cata-gelatin gel shows, however, a higher figure than either, pointing to structural difference. The insoluble ana-gelatin is more labile than the soluble,

since on standing in 18 % acid before hydrolysis, the basic nitrogen is definitely increased.

Table XII.

	Amide-N	Humin-N	Diamino-N	Mono- + non-amino-N	Total N
Precipitate from flocculation in alkaline solution	1·8	0	23·2	75·3	100·3
Ditto, after six washings	1·6	0·1	23·5	74·9	100·1
Ditto, after standing 24 hours in 18 % HCl	1·5	0	25·9	72·7	100·1
Cata-gelatin gel	1·5	0	29·2	69·2	99·9

Two differences are thus observed between the soluble and insoluble ana-fractions; the amide-nitrogen, which is nearly twice as high in the former case, and the diamino-nitrogen, which is 4 % higher in the latter. The insoluble cata-gelatin has the same amide-nitrogen as the insoluble ana-gelatin, but a higher diamino-nitrogen than either of the ana-gelatins.

(In experiments upon the effect of acid and alkali treatment of the precursor of gelatin, it was found that the diamino-nitrogen of the precipitated portion, after alkali treatment, was 24·7, and of the supernatant liquid (corresponding with the soluble fraction) about 18 %. These numbers show close agreement with those above, which may thus give a clue to the effect of precursor treatment [Knaggs and Schryver, 1924, 3].)

Series K. (*Experiments by Dr A. B. P. Page.*)

Effect of heat treatment of purified gelatin. Within the somewhat large limits of error in the Van Slyke method for determination of amino-nitrogen there was no change in the amino-nitrogen after heating for 20 hours at 100°.

Electrometric formaldehyde titration. The heated gelatin was diluted 1 : 1 before titration. Titrations were then made until the E.M.F., using a hydrogen electrode, reached 650 millivolts—the limit beyond which the electrodes became unreliable in the presence of formalin.

Titre of the 25 cc. formalin	2·95 cc. NaOH	
Titre of 25 cc. unheated 2 % gelatin	9·25 − 2·95 = 6·3	cc. NaOH
Titre of 25 cc. unheated 1 % gelatin	3·13	,,
Titre of 25 cc. heated 1 % gelatin	6·50 − 2·95 = 3·55	,,
Hence difference due to heating	0·42	,,
This is equivalent to	0·29	mg. nitrogen
25 cc. heated 1 % gelatin contained	42·0	,,
Hence % increase of amino-N on heating a 2 % solution for 21 hours at 100°	0·69 %	

Fractional dialysis. 300 cc. of a 1·85 % solution of a once flocculated gelatin were heated for 5 hours at 100°. 200 cc. were used for dialysis. The collodion membranes were poured on a glass plate from 5 % alcohol-ether solution of necolloidine, and covered with a Petri dish. They were air-dried, soaked overnight in alcohol of the required strength and finally washed for 3 or 4 hours in frequent changes of distilled water.

Two or three changes of dialysis water were made with each membrane, and the nitrogen determined in each. The amount of nitrogen which diffused in the time, and its percentage of the total nitrogen are given below.

Table XIII.

Membrane soaked in	Time of dialysis (hours)	Total N diffused (mg.)	% of total N
60 % alcohol	21	0·39	0·06
	Subsequent 67	0	0
	,, 89	0	0
	Total 177	0·39	0·06
80 % alcohol	16	2·0	0·32
	Subsequent 40	0·5	0·08
	,, 84	0·4	0·06
	Total 140	2·9	0·46
90 % alcohol	25	0·75	0·12
	Subsequent 41	0	0
	Total 66	0·75	0·12

Hence the total nitrogen diffused in 383 hours = 0·64 %. This agrees with the change in amino-nitrogen, and also with the diffusible nitrogen found for purified gelatin in Series A, Part 2.

Summary.

1. Evidence has been given to show that the molecules, or aggregates of molecules, of which gelatin is composed readily undergo intramolecular change.

2. This evidence suggested the possibility that the intramolecular arrangement was different according to whether gelatin was functioning as anion or cation.

3. By the electrolysis of the hydrochloride, gelatin was obtained in the form of a gel, with very little nitrogen in the supernatant fluid. By the electrolysis of the sodium salt, two fractions were obtained, *viz.* an insoluble fraction (insol. ana-gelatin) and a soluble fraction (sol. ana-gelatin). Both were complex proteins. Insoluble ana-gelatin is soluble in water to the extent of about 15 parts in 100,000 at 15°; soluble ana-gelatin has a molecular weight of 50,000–60,000.

4. Attempts were made to resynthesise a gel by combination of the soluble and insoluble ana-gelatins, but without success.

5. Two possible relationships between the soluble and insoluble proteins into which gelatin is separated on electrolysis of the sodium salt are suggested:

(*a*) that they are independent proteins having no relationship to one another other than their association together in the gelatin complex;

(*b*) that the soluble fraction is a product of the action of water on an insoluble gelatin. The combination of a soluble and insoluble fraction in some form is responsible for the gel formation.

On electrolysis of the sodium salt the two fractions separate, undergoing in the process intramolecular change, which prevents their recombination.

6. Evidence is given to show that by the action of water on gelatin a product is produced which is a highly complex protein, and that the change is not accompanied by any appreciable amount of peptide scission. For the changed gelatin the term *meta-gelatin* is suggested. If the second of the above conceptions is correct, the gel called cata-gelatin is a close combination of gelatin and meta-gelatin, which separate on electrolysis of their sodium salts, undergoing at the same time an intramolecular change. There is also evidence that insoluble gelatin cannot be dispersed in water without undergoing a slight irreversible change and conversion to a soluble form.

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CLXXI. INVESTIGATIONS ON GELATIN.

PART X. THE EFFECT OF PREVIOUS TREATMENT WITH ACID AND ALKALI ON THE DIAMINO-NITROGEN FRACTION OF THE HYDROLYSIS PRODUCTS OF GELATIN.

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In these experiments Coignet's Gold Label gelatin purified by electrodialysis was used. Gelatin was allowed to stand in contact with HCl solutions varying in strength from concentrated acid to $N/70$. The most convenient quantity of gelatin for determining the Hausmann numbers by the micro-method [Thimann, 1926] is 0·1 g. of the dry protein, and this amount was taken throughout. After standing for 48 hours at room temperature the solutions were hydrolysed by pouring into boiling acid at such a rate that the boiling was not interrupted. The acid in the flask was of such a strength that the final mixture was approximately 20 %.

A few duplicate experiments were performed allowing a time of 3 days before hydrolysing instead of 48 hours. As the results agreed fairly closely it was assumed that 48 hours was sufficient for equilibrium to be reached.

Table I (see also Fig. 1).

Concentration of HCl solution	Amide-N %	Diamino-N %	Mono-+ non-amino-N %	Total %
0	2·6	17·7	79·6	99·9
0·0143 *N*	2·4	17·8	79·8	100·0
0·1 *N*	2·7	18·4	79·1	100·2
0·77 *N*	2·4	20·6	77·2	100·2
1·4 *N*	2·3	22·3	75·2	99·8
2 *N*	2·5	23·0	74·3	99·8
2·3 *N*	2·4	23·5	73·8	99·7
3·45 *N*	2·5	22·9	74·6	100·0
5·45 *N*	2·3	21·3	76·1	99·7
8·8 *N*	2·2	15·2	82·3	99·7

Effect of NaOH *solutions.*

A similar series of experiments was carried out with gelatin in the presence of alkali. As the presence of salt influences the diamino-nitrogen precipitated by phosphotungstic acid, control experiments were performed. The hydrolysing acid in these cases contained exactly the same amount of salt as the acid used in the hydrolysis of the protein solutions.

Table II.

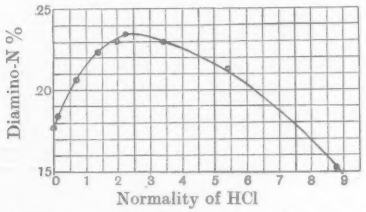
Concentration of NaOH	Amide-N %	Diamino-N %	Mono-+ non-amino-N %	Total %
0·1 *N*	2·2	18·0	79·3	99·5
Control 0·1 *N*	2·5	18·1	78·8	99·4
N	2·8	21·0	76·7	100·5
Control *N*	2·6	18·6	78·5	99·7
2 *N*	2·6	23·6	73·4	99·6
Control 2 *N*	2·3	19·1	78·9	100·3

Hence the corrected values of diamino-nitrogen due to the influence of NaOH are as follows, the excess over 18 in the controls being subtracted from the experimental numbers:

Normality	0·1 *N*	*N*	2 *N*
Diamino-N	18·0	20·4	22·5

Effect of acid on heated gelatin.

A 10 % gelatin solution was boiled in a steamed-out Jena flask for 2 days; 1 cc. of this solution was allowed to stand with 10 % HCl solution, which is the concentration which gives the greatest rise of diamino-nitrogen with ordinary unheated gelatin.



Gelatin treated with HCl prior to hydrolysis

Fig. 1.

Table III.

	Amide-N %	Diamino-N %	Mono-+ non-amino-N %	Total %
Heated gelatin	2·5	18·5	79·5	100·5
Treated with 10 % HCl	2·6	18·1	79·0	99·7

Effect of pepsin and trypsin.

200 cc. of 4 % gelatin solution in *N*/10 HCl were warmed to 37° and 5 cc. of 1 % pepsin solution added. This mixture was kept at 37° and at intervals 25 cc. were pipetted into a flask, cooled and titrated with *N*/10 NaOH in the presence of formalin. When the formalin titration values became constant a portion of the gelatin solution was hydrolysed and the Hausmann numbers were determined. At the same time a control experiment was set up using the same quantities of reagents but the activity of the pepsin was destroyed by boiling before it was added to the gelatin solution.

A similar experiment was performed with trypsin.

Table IV.

	Amide-N %	Diamino-N %	Mono-+ non-amino-N %	Total %
Pepsin	2·9	19·3	77·5	99·7
Control	2·4	19·0	78·2	99·6
Trypsin	2·5	20·0	77·0	99·5
Control	2·6	19·2	78·4	100·2

Hence the corrected values of diamino-N due to enzyme action are as follows:

Pepsin	...	...	18·3
Trypsin	...	...	18·8

The gelatin used had not been freed from the soluble fractions by flocculation, and therefore the numbers in this paper are not absolute; they do, however, indicate the general trend of the action of acids and alkalis on gelatin.

This work was carried out on the suggestion of Prof. S. B. Schryver.

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CLXXII. COMBINATION OF PROTEINS WITH PHTHALEIN DYES.

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Many properties of proteins have been ascribed to adsorption phenomena and surface effects—grouped under the name of colloidal properties. Loeb [1922] and others have shown that many properties of proteins may be explained by the orthodox laws of chemical combination. In this paper an attempt is made to explain the effect of proteins on phthalein dyes as a chemical phenomenon.

The interesting observation was made by Rosenthal [1926] that when a small amount of protein has been added to a dilute rose bengal solution the deep pink colour is not discharged as usual when the solution is acidified. This he ascribes to the fact that the dye behaves as though it were not in solution. It was thought that this observation deserved further attention and it was found that if the colour of a dilute rose bengal solution were discharged by acidification with hydrochloric acid and then a small amount of protein were added and the solution warmed, a rose-red colour soon developed. The combination of protein and dye resulted in colour change, so that the simple explanation of the dye behaving as though it were not in solution is hardly accurate since the same colour is obtained when the dye is undoubtedly in solution and already decolorised. The reaction between the protein and acidified dye proceeds very slowly in the cold, probably because an intramolecular change takes place as will be shown later.

The question arose as to whether rose bengal (tetraiodotetrachlorofluorescein) was unique in its behaviour or whether all fluorescein and phenolphthalein derivatives behaved similarly. The first dyes examined fell into two classes:

(I) *Colour discharge prevented by protein.*

Rose bengal, eosin (tetrabromofluorescein), erythrosin (iodofluorescein) and phloxin red (dichlorotetrabromofluorescein).

(II) *Colour discharged even when protein is present.*

Phenolphthalein, tetrachlorophenolphthalein, tetrabromophenolphthalein, phenolsulphonephthalein and bromothymol blue.

It would thus seem that fluorescein derivatives behaved in one way and phenolphthaleins and sulphonephthaleins in the other. But the deep yellow colour of alkaline fluorescein solutions changed to the pale yellow colour when

acidified even when protein was present. Thus some other explanation was necessary since fluorescein behaves differently from its derivatives. It was noticed that the usual change of colour of all the dyes of class I takes place at reactions on the acid side of the isoelectric point of the protein (p_H 4·8), while the usual change of colour of dyes of class II occurs on the alkaline side of the isoelectric point. The behaviour of thymol blue (thymolsulphonephthalein) supports this observation. This dye changes from blue to yellow in alkaline solution (p_H 9·6) and from yellow to red in acid solution (p_H 2·8). When protein was added the change from blue to yellow on adding acid was not interfered with, but the change from yellow to red did not take place when small amounts of serum-proteins were present before acid was added. The p_H at which the dye changes colour, therefore, determines its behaviour.

Table I. *Reaction of colour change of dyes.*

Reaction at which colour begins to change when acid is added.			
Colour retained in acid protein solution		Colour discharged by acidification	
Dye	p_H	Dye	p_H
Eosin	3·0	Thymol blue—alkaline range	9·6
Erythrosin	3·6	Fluorescein	5·6
Phloxin red	3·6	Bromothymol blue	7·6
Rose bengal	4·6	Phenolsulphonephthalein ...	8·4
Bromophenol blue ...	4·6	Tetrabromophenolphthalein	9·0
Thymol blue—acid range	2·8	Tetrachlorophenolphthalein	9·4
		Phenolphthalein	10·0

From this it is evident that acid protein ($PrNH_3^{\cdot} + Cl'$) and coloured (quinonoid) dye must co-exist if colour discharge on acidification is to be prevented. Now Meyer and Hantzsch [1907] describe complexes of phthalein dyes and heavy metal salts (*e.g.* $SnCl_4$) which are red in colour, and therefore have the quinonoid structure, and are probably oxonium salts of the type I.

It is suggested therefore that we are dealing with dye-protein chloride compounds of the type II. Very strong support for this view is found in the fact that alkaloids similarly interfere with the colour discharge of certain phthalein dyes [Messner, 1903], as does benzidine, as pointed out to me by Dr Marrack. In the case of simple aromatic amines there can be little doubt that the mechanism is chemical combination and it seems highly probable that the same applies to proteins and dyes.

Further evidence for the view of oxonium salt formation is found in the fact that the colour change of rhodamine B is not prevented by adding protein before acidifying. Rhodamine B changes colour on the acid side of the isoelectric point of proteins (p_H 1·2) but the dye does not possess a quinonoid structure and the chromophoric group is a diethylimino-group.

Fluorescent dyestuffs no longer fluoresce when combined with protein in acid solution. When serum-albumin and acid are added to eosin and erythrosin the colour deepens, so that the acid solution is actually more deeply coloured than the original alkaline solution. In addition to this the tint is altered on adding protein and acid. The absorption spectra of the compounds were therefore studied. The author is indebted to Prof. Roaf for allowing him to use his spectrometer and calibrations.

Table II. *Absorption bands.*

Wave lengths in Ångström units.

Dye		Alkaline dye		Acid-protein-dye		Shift in band
		Limits	Mean	Limits	Mean	
Rose bengal (0·01 %)	1st band	4978–5186	5080	5220–5360	5290	210
	2nd band	5378–5522	5450	5580–5700	5640	190
Eosin (0·008 %)		5000–5270	5135	5290–5490	5390	255
Erythrosin		4980–5300	5140	5290–5480	5395	255
Phloxin	1st band	5047–5175	5111	—	—	—
	2nd band	5313–5453	5383	5443–5615	5529	146

These dyes when acidified possessed no prominent bands in the visible region of the spectrum. There is thus a shift of 150–250 Å. in the absorption bands when protein and hydrochloric acid are added to an alkaline dye. So marked an alteration in the absorption spectrum suggests the formation of a new chemical compound with properties different from its components rather than loose physical attachment or adsorption of the dye by protein micellae. One might note at this point that Rosenthal assumed that the colour of rose bengal did not change when combined with acid protein and calculated the amount of dye combined by comparison of the colour of the acid-protein dye with that of alkaline rose bengal. As there is certainly a change in tint and probably in intensity of the colour this seems hardly justifiable. His method of protein determination in the cerebrospinal fluid by this method may also be criticised on the ground that a very large difference in protein concentration has a very small effect on the colour produced.

As we have seen, closely defined conditions as to the critical reactions of the dye and protein must be observed in order that the phenomenon of colour retention in acid solution should occur. This specificity supports the view of chemical combination, rather than physical adsorption. Further support for this view is found in the shift of the absorption band which one would not expect with a purely surface effect.

If the combination of dye and protein were purely an adsorption phenomenon and surface effect it would be expected that surface-active substances, *i.e.* those lowering interfacial surface tension, would have a great effect on the combination. Ten liquids not miscible with water were chosen, viz. amyl and capryl alcohols, chloroform, ether, liquid paraffin, oleic acid, olive oil, light petroleum, toluene and turpentine. The interfacial tension of these liquids against water varies from about 4 to 40 dynes per cm. [Du Noüy,

1926], and one would expect differences in their effect on an adsorption system. It was found, however, that none of the liquids had any appreciable effect on the combination of eosin with proteins whether the liquid was shaken with the acid-protein-dye solution, or with the eosin before the protein and acid were added, except that some of the liquids extracted a small amount of acid-eosin.

Rosenthal [1925] states that 10 mg. of sodium taurocholate prevented the combination of rose bengal with one drop of serum (*i.e.* about 3 mg. of protein). Apart from the fact that this seems an excessive amount of bile salt to have to use if a pure surface effect were involved, it was found that if 10 mg. of sodium taurocholate or saponin were added to one drop of horse serum, and then 10 cc. of rose bengal and 4 cc. of $N/10$ hydrochloric acid were added, the solution remained deep pink in colour. The colour of the solution had been slightly reduced by the surface-active substance but the combination was only very partially inhibited. Possibly the result described by Rosenthal was due to the effect of the bile salt on the membranes he used. The absence of any very great effect of surface-active substances on the dye-protein combination is further evidence that the effect is not purely surface adsorption.

When considerable amounts of concentrated acids, *e.g.* 30 % hydrochloric acid, 20 % salicylsulphonic acid, are added to the acid-protein-dye solutions the colour is discharged, probably because the dye cannot exist in the coloured quinonoid form, even when combined with protein, in strong acid solution. In the case of salicylsulphonic acid the protein is, of course, precipitated, but, even when the sparse precipitate is dissolved by warming, the colour is not regained.

In all these experiments serum, serum-albumin and globulin, egg-albumin and gelatin behave similarly except that gelatin manifests less dye-combining power, possibly due to the molecular aggregation of this protein as suggested by Rosenthal [1926].

The main points supporting the view of chemical combination between the dyes and protein rather than the idea of surface adsorption are as follows.

(1) The specificity and rigidly defined conditions of p_H, etc., for combination to occur.

(2) The analogy between proteins and simple organic bases in their behaviour towards dyes.

(3) The shift in absorption band which suggests a chemical rearrangement rather than loose physical forces being called into play.

(4) The lack of effect of surface-active substances on the combination.

Small strips of silk and wool were immersed in fairly concentrated (*ca.* 1 %) alkaline solutions of the dyes, the temperature was rapidly raised to the boiling-point, the solutions were cooled and the material immediately removed from the bath, rinsed with water and immersed in $N/10$ hydrochloric acid. The results were as follows.

Dye	Silk	Wool
Eosin	Deep pink	Scarlet
Erythrosin	Magenta	Deep red
Rose bengal	Magenta	Deep magenta
Thymol blue	Yellow	Deep yellow
Phenolphthalein	Colourless	Colourless
Fluorescein	Colourless	Almost colourless

These results are thus in complete accord with those obtained with serum-proteins, egg-albumin and gelatin. Those dyes which give coloured compounds with the proteins in acid solution dye the fabrics and the dye is acid-fast, whilst dyes which do not give coloured solutions with acid-proteins do not dye the fabrics to any extent.

Similarly it was found when paraffin sections of various tissues were stained that the same tissues were stained by eosin, erythrosin and rose bengal, and the differences in tint with the different dyes were similar to those with wool and silk. When the stained sections were immersed in $N/20$ hydrochloric acid the stain was not removed. Evidently this histological protein staining is another manifestation of the chemical phenomenon described. Differential staining by treating with acids after the phthalein dyes is therefore due to the stability of the protein-dye compound in acid solution.

Rous [1925] has injected phthalein dyes into living animals and from the colour assumed by the various organs, draws important conclusions regarding the p_H of different tissues. The greatest caution should be exercised in drawing such conclusions since many of the phthalein dyes combine with proteins, and the tint of some of these compounds is slightly different from that of the dye itself, and the colour of the compound varies little with change of p_H.

It will be obvious also that many of these dyes cannot be used as indicators as the "protein error" is very great. The case of bromophenol blue may be cited as an example. In series I, one drop of the dye solution was added to 5 cc. of acetate buffer of each p_H. In series II, a small amount of serum was added to the dye solution, and one drop of the alkaline dye-protein solution was added to each buffer.

	p_H	3·2	3·4	3·6	3·8	4·0	4·2	4·4	4·6
I. Dye alone	...	yellow		green-yellow	green-blue			blue-violet	
II. Protein present		green-blue			blue				

The tints of the tubes of series II are in no case the same as those of series I and are deeper, but apart from this not even an approximate idea of the p_H can be gained from the colour of the tubes of series II. If to each of the dye-buffer solutions of series I one drop of ten times diluted serum is added the colours alter slightly, but even after 24 hours' standing at room temperature the colours are not the same as those of series II; but if the tubes are plunged in a boiling water-bath for a few seconds and then cooled, the colours of series I and II are indistinguishable. In the tubes of series II, the dye is in the quinonoid form when brought into contact with the acid protein, hence coloured

oxonium salt formation occurs immediately. In the tubes of series I, however, the acid protein is brought into contact with the dye, mainly in the phenolic form, hence combination is slow, but on warming some of the phenolic form changes into the quinonoid form and this combines with the acid protein. Change of phenolic into quinonoid dye in the cold is slow under these conditions but when warmed more quinonoid dye is formed—if a greenish solution of bromophenol blue is warmed the solution becomes blue while hot, but greenish again on cooling. The formation of some quinonoid dye from phenolic dye on heating is a property of many phthalein dyes.

The dyes bromophenol blue, rose bengal, phloxin red, erythrosin, eosin and thymol blue (the last-named in its acid range) cannot therefore be used as indicators in the presence of proteins. Since combination of the protein occurs only with the quinonoid dye, different end-points are obtained when solutions containing protein are titrated, using these dyes as indicators, according to whether acid is added to the alkaline solution or alkali to the acid solution.

In connection with these phenomena an observation of Deutsch [1927] is of interest. He observes that when an aqueous solution of thymol blue, just on the alkaline side of its range of colour-change from the yellow quinonoid to the red phenolic form, is shaken with benzene, a reddish violet colour appears, which disappears when the liquid settles down. This he ascribes to alteration of the dissociation constant of the dye when adsorbed at the benzene-water interface. There is no evidence for this assumption and another explanation is possible. Only the phenolic red form of thymol blue is soluble in benzene in which it gives a reddish violet solution. It is probable therefore that only the phenolic form is adsorbed at the benzene-water interface. In the solution used by Deutsch there is a small amount of the red form in equilibrium with a large amount of the yellow form. When the fluid is shaken the benzene-water interface is greatly increased and all the red form present is adsorbed; some of the yellow form in the aqueous solution then changes over to the red form, to maintain the equilibrium in the water, and this is promptly adsorbed and so on, the reddish colour of the dye adsorbed at the interface predominating over the yellow colour of the aqueous solution. When allowed to settle down the adsorption of the red form becomes negligible since the interface has decreased enormously, and when it passes into the water it is mostly changed to the yellow quinonoid form to give the original equilibrium. There is therefore no need to postulate a change in dissociation constant to explain the behaviour of the dye.

Up to now we have been dealing with very dilute solutions of dyes (*ca.* 0·01 %). When more concentrated solutions are employed another phenomenon has been observed. If serum-proteins are added to a fairly concentrated aqueous dye solution, *e.g.* 0·25 % eosin, and then dilute hydrochloric acid is added gradually, a reddish precipitate first forms and then, on adding more acid, the precipitate dissolves, giving a clear, deeply-coloured solution. If the dye alone is acidified a yellow precipitate is formed and this does not dissolve

in excess acid. This behaviour is not confined to the dyes which remain coloured in acidified protein solutions, but occurs with all the dyes investigated, *i.e.* phenolphthalein and its derivatives, phenolsulphonephthalein and its derivatives, and fluorescein and its derivatives. Those dyes which do not remain deeply coloured in acidified protein solutions do not give deeply coloured precipitates, *e.g.* phenolphthalein gives a colourless precipitate soluble in excess acid. Serum, serum-albumin, serum-globulin, egg-albumin and gelatin all behave in the same way when sufficient protein is added. When an insufficient amount of protein is employed a clear solution in excess acid is not obtained. The precipitation of yellow phenolsulphonephthalein-serum-albumin occurred, although both the dye and the protein alone are soluble at all reactions.

Electrometric determinations were carried out with Dr J. R. Marrack's apparatus and help to determine the reactions at which precipitation and solution occurred. Precipitation commenced near the isoelectric point of the protein, *i.e.* when the protein began to function as a base, and the precipitate dissolved, giving a clear solution at about p_H 2·5. These points appeared to be independent of the nature of the dye.

	Precipitation		
Dye change-point	Began	Cleared	Clear
p_H	p_H	p_H	p_H
3 eosin—serum-albumin	4·9	2·9	2·3
3 eosin—gelatin	4·7	—	—
8 phenol red—serum-albumin	4·5	—	2·6

In three cases analysis of the protein-dye precipitates was conducted as follows: The protein was added to the alkaline dye solution and acid was added until a copious precipitate formed. This was allowed to stand overnight and was then filtered off on a hard filter-paper on a Büchner funnel. The dye precipitate was then dissolved in $N/10$ hydrochloric acid, the protein determined by Kjeldahl's method and the dye determined by adding 1 cc. of the solution to hot alcohol, making up the volume to 25 cc. and then comparing the colour of the alcoholic solution, when made alkaline, with that of standard alcoholic solutions of the dyes.

Constituents	Dye %	Protein %	Mols. dye / Mols. protein
Eosin—serum-albumin	0·27	0·85	20 : 1
Rose bengal—serum-albumin	1·765	2·26	34 : 1
Phenol red—egg-albumin*	1·1	4·66	28 : 1

* Assuming the molecular weight of egg-albumin to be 44,000 [Marrack and Hewitt].

It seems probable that these precipitates are due to salt formation between the dyes which have acidic properties and the proteins which on the acid side of the isoelectric point behave as bases. Chapman, Greenberg and Schmidt [1927] describe the formation of similar insoluble protein-azine-dye precipitates. The varying ratio of dye molecules to protein molecules is due to the fact that at these reactions the maximum acid-binding power of the protein is not reached. The solution of the protein-dye compound in excess acid is probably

due to the combination of the basic groups of proteins, which are not already combined with dye, with hydrochloric acid forming soluble hydrochlorides. In support of this is the observation that when excess dye is present and nearly all the basic groups are combined with dye the compound does not dissolve in excess acid.

Since the acidic groups of the dyes at these reactions are mainly phenolic it would be anticipated that phenol itself would behave similarly to the dyes, and this was found to be the case. When a small amount of protein is added to a dilute solution of phenol and acid is gradually added, a precipitate first forms and this dissolves up when more acid is added. A typical experiment with acetate buffers was as follows:

p_H	5·2	5·0	4·8	4·6	4·4	4·2
Precipitation	—	±	+ + +	+ +	+ +	–

Hoffman and Gortner [1925] suggest that on the acid side of p_H 2·5 proteins do not combine chemically with acids but form adsorption compounds. Chapman, Greenberg and Schmidt [1927] find that in solutions more acid than p_H 2·5 certain dyes combine with proteins in stoicheiometric proportions giving protein-dye precipitates. The valency of the proteins in these compounds approximates closely to the maximum acid-combining power of the proteins calculated from their diamino-acid content. Gortner's [1927] reply is that these results "offer very striking substantiation of the conclusions reached by Hoffmann and Gortner." Gortner assumes that in very acid solution there are present protein cations with surface electrical charges and the number of these charges is equal to the chemical valency of the protein, and the same with the dye. These charges are neutralised and a protein-dye colloid is precipitated. It seems probable, however, that such neutralisation of ionic charges may well be chemical combination, and is in no wise different from the precipitation of barium sulphate by the neutralisation of the ionic charges of barium and sulphate ions and formation of an insoluble chemical compound. The mere fact of the protein cation being larger than the barium cation does not change a phenomenon from chemical combination to physical adsorption.

Summary.

Phthalein and fluorescein dyes which change colour on the acid side of the isoelectric point of proteins combine with proteins in acid solution giving coloured oxonium salts. The colour of such dyes is not discharged or altered as usual on acidification and hence they cannot be used as indicators in solutions containing proteins. The "protein error" of indicators, acid-fast staining of tissue sections and acid-fast dyeing of fabrics are explained.

When any phthalein or fluorescein dye is mixed with a protein solution and acid is added, a precipitate forms at the isoelectric point of the protein and dissolves at about p_H 2·5. Salt formation is indicated, and it is considered unnecessary to postulate physical adsorption to explain any of the phenomena described.

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CLXXIII. THE SURFACE TENSION OF PROTEIN SOLUTIONS. PART III.

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The surface tension of protein solutions has been given a definite meaning by the work of du Noüy [1926] who has shown strikingly the adsorption of the protein molecule at the surface, forming there an organised unimolecular layer similar in every respect to the orientated unimolecular films of fatty acids and other long chain organic compounds of Langmuir and Adam. Du Noüy's calculations of the dimensions of the egg-albumin molecule from surface tension measurements, which give a value for molecular weight in close agreement with those of other observers, are further evidence that the colloidal behaviour of protein solutions is due to the size of the single molecule, although agglomeration into micellae may occur under certain conditions. Thus, for a given concentration, volume of solution and area of surface, any alteration, chemical or physical, in the molecule will affect the surface tension by the alteration of the surface layer.

In previous communications [1925, 1926] the Donnan equilibrium was considered to affect the surface tension of a protein solution according to the Lippmann effect of the charge on the particle, which Loeb [1922, 1] has proved to be due to the potential difference between the particle and the solution. In view of du Noüy's researches establishing the existence of an organised surface layer in protein solutions, it can be seen that the greater the charge on the molecules of protein, the greater the repulsive forces between adjacent molecules, hence the lower the cohesive forces and the tensile strength of the surface layer.

The earlier work on surface tension of gelatin solutions showed, however, that this is not the only factor affecting the surface tension of protein solutions, the solubility, hydration and denaturation of the protein molecule also being important influences independent of a membrane equilibrium which will alter the manner of the packing of the surface layer.

In the present communication the effects on the surface tension of an alteration in the protein molecule due to solubility, change of reaction, presence of neutral salts, and denaturation were studied for caseinogen and egg-albumin in order to extend the previous work of St Johnston and Peard [1925, 1926].

Experimental.

The method used both in the present and previous work for the determination of the surface tension was the maximum bubble pressure method of Sugden [1922, 1924]. This method, together with hanging-drop methods of which it is the inverse, is open to the criticism that it is comparative only. Du Noüy [1926] has also shown the importance of the time factor in the determination of the surface tension of colloidal solutions due to the slow rate of adsorption of the particles or molecules at the surface; he states, "It is thus essential when giving the surface tension of a colloidal solution to state the time which has elapsed since the solution was violently agitated, and, unless the method is a true static one, for results to be even comparable amongst themselves, everything being otherwise equal, the time required for the formation of a drop or bubble must be about the same for a large number of drops." Wolf [1923] has shown for bacteriological media that the surface tension as determined by a modification of du Noüy's static ring method is about 10 dynes lower than that determined by the drop method with a fast rate of dropping, whereas when the time of dropping was much prolonged agreement between the two methods was close.

The bubble method employed in this investigation was carried out under standard conditions with an approximately constant bubble rate, and the mean of several readings taken for each determination. Since the solutions employed were relatively concentrated compared to those of du Noüy, equilibrium at the surface would be attained much more rapidly owing to the more rapid diffusion towards the surface layer. Again, the pressure difference between two jets is observed; hence the effect of small variations in the bubble rate is partly compensated.

A further objection to Sugden's method when applied to colloidal solutions has been made by King [1925] on account of the frothing due to the bursting of the bubbles. Peard and St Johnston [1925], however, have shown for brewery worts that the amount of protein removed in this manner is negligible in the course of a determination of the surface tension, whilst frothing at the surface of the solution may be very much reduced or entirely prevented by the presence of a trace of toluene there without affecting the surface tension at the fresh bubble surface formed within the bulk of the liquid.

The determinations were carried out at 25° and the solutions were prepared 24 hours beforehand and kept at 25° to ensure chemical equilibrium being attained, a drop of toluene being added to ensure sterility and to prevent frothing. The bubble rate on the small jet was timed for each reading and was at a rate of one bubble in 1–3 seconds, but no appreciable constant difference was found experimentally between the pressure-difference readings for one bubble in one second and one in five seconds. The mean of five or six readings at various bubble rates within the above limits was taken as the value of the surface tension required. All the instruments used had jets of

approximately the same size, were calibrated with pure benzene and checked with water; the pressure difference readings for water were from 7 to 11 cm. on the alcohol gauge for various instruments, and all gave values for the surface tension of water within 0·5 %. They also agreed with each other to that extent when checked with the same protein solution. All the containers used were cleaned with chromic acid and washed and dried. Corks or rubber bungs were covered with tinfoil. The surface tension instruments were cleaned with nitric acid and well washed between each determination. In the preparation of solutions only boiled distilled water kept sterile and CO_2-free was used, and for alkaline solutions a small soda-lime tube was attached to the bubbler of the surface tension instrument to prevent any access of CO_2 from the air. 5 cc. of solution were required for a determination.

The p_H determinations below p_H 7·5 were made at 20° with the quinhydrone electrode, using a gold electrode according to the technique of Corran and Lewis [1924] and 0·03 g. ground quinhydrone crystals per 20 cc. of solution. Above p_H 7·5 the determinations were made with the hydrogen electrode.

The caseinogen used was a commercial pure preparation which was fat-free and contained 9·58 % moisture, 0·11 % ash and 14 % N. It was therefore deemed suitable for use without further purification, being ash-free except for that organically combined, and, taking 6·47 as the protein factor, it contained 90·6 % caseinogen. In all experiments 0·56 g. of this caseinogen was dissolved in 50 cc. of solution containing the requisite quantity of $N/10$ acid or alkali to give a 1 % solution.

The crystallised egg-albumin was prepared from new-laid eggs according to the method of Sørensen [1915], recrystallised twice and dialysed for 3 weeks at 5°. Owing to the somewhat prolonged dialysis and slight leaks, the resulting albumin solutions were only about 0·6 %, so that when diluted with the requisite amount of $N/10$ acid or alkali they were reduced to 0·3 %. This was, however, sufficient to give a marked lowering of surface tension. Solution A contained 0·92 mg. protein-N and 0·016 mg. ammonium sulphate-N per cc. and had p_H 5·6; it was faintly turbid. Solution B contained 0·96 mg. protein-N and 0·036 mg. ammonium sulphate-N per cc. and had p_H 6·76; it was quite clear.

The effect of time on the surface tension of caseinogen solutions.

Owing to the low solubility of caseinogen, a considerable time was required for solution to take place, even when the conditions were favourable for complete solution. It was therefore necessary to determine the effect of the age of the solution on the surface tension, in order that future determinations of surface tension should be made when equilibrium had been attained. Caseinogen will only give completely dissolved 1 % solutions with hydrochloric and phosphoric acids between p_H 3·0 and 1·6, and with sodium hydroxide of p_H greater than 7·5. The effect of time on the surface tension of 1 % caseinogen solutions in HCl at p_H 2·46 is shown in Table I. Solution in

acids occurs with swelling and there is a rapid fall of surface tension in the first few minutes followed by a slower fall during about 24 hours, after which there is little change. The solutions were opalescent at first and did not clear for about 12 hours. Similarly with a partial solution in sodium hydroxide, p_H 6·8, equilibrium was not reached until nearly 24 hours had elapsed, although in alkali no swelling occurs.

Henceforth all determinations of surface tension of 1 % caseinogen solutions or suspensions were made 24 hours after preparation.

Table I. *Effect of age of solution of caseinogen on the surface tension.*

1 % caseinogen in $N/100$ HCl at 25° (2 experiments)			1 % caseinogen in $N/250$ NaOH at 25°	
	Surface tension			
Time	(*a*) Dynes per cm.	(*b*) Dynes per cm.	Time	Surface tension Dynes per cm.
2 mins.	71·5	64·9	2 mins.	70·8
4 „	70·8	62·6	5 „	60·1
6 „	70·2	61·5	8 „	59·6
10 „	69·5	60·5	12 „	59·0
15 „	63·1	60·5	15 „	58·6
20 „	61·9	—	30 „	58·1
50 „	61·5	—	45 „	58·0
60 „	61·0	59·7	60 „	58·0
2 hours	60·5	59·2	2 hours	58·0
6 „	59·5	—	19 „	57·0
18 „	59·0	58·5	26 „	56·9
24 „	58·7	58·6	43 „	56·3
43 „	—	58·5		

The effect of p_H on the surface tension of caseinogen solutions.

The titration curves of caseinogen in presence of HCl and H_3PO_4 were identical with those of Loeb [1922, 1], but the curve obtained for caseinogen in presence of $N/10$ NaOH differed from Loeb's, being always less alkaline for a given amount of NaOH added, particularly on the part of the curve where the change of p_H with increased addition of alkali is most rapid, *i.e.* between p_H 6 and 10. A regular curve was however obtained, and values of p_H determined with the hydrogen electrode agreed well with those determined by the quinhydrone electrode up to the limit where the use of the latter is possible. A clear 1 % solution of caseinogen in NaOH was not obtained below p_H 7·5. The rapid hydrolysis of caseinogen by alkali when the p_H is greater than 10 where a change in direction of the titration curve is apparent has been pointed out by Zoller [1921]. It was noted that solution took place very rapidly in liquids alkaline to p_H 10 and that all power of "head" retention was lost, foam formed on shaking disappearing immediately. The results of the variation of the surface tension with p_H are shown in Fig. 1. It will be seen that the general form of the curve is similar to that obtained by St Johnston and Peard [1925, 1926] for gelatin, the maximum surface tension being at the iso-electric point, p_H 4·62, as expected from Donnan's theory, though for caseinogen the value did not rise to that of pure water as in the case of gelatin, being 67·8 dynes per cm. Robertson [1918] and Cohn and Hendry [1923] have,

however, shown that caseinogen is slightly soluble in water when pure, even at the isoelectric point. The fall of surface tension is very rapid on either side of the isoelectric point and there is a minimum of surface tension on the acid side at p_H 3·8–3·9 followed by a well-marked secondary maximum at p_H 3·4, such as was obtained for gelatin at p_H 2·0–2·2. This maximum at p_H 3·4 is just before the caseinogen becomes completely soluble (about p_H 3·0). In presence of H_3PO_4 the surface tension proceeds parallel to that in presence of HCl but is uniformly a dyne lower. The maximal values of the osmotic pressure and viscosity of caseinogen in presence of HCl are at p_H 3·0, and Zoller [1921] states that "acid hydrolysis of caseinogen must of necessity commence somewhere in the neighbourhood of this drop or flattening of the curve." St Johnston and Peard [1925], however, were unable to find in the case of gelatin solutions acid to p_H 2·2 any evidence of hydrolysis as evinced by formaldehyde titration commencing suddenly below p_H 2·2. This point is dealt with later. The minimum value p_H 8·5 agrees fairly well with Zoller's value of p_H 9·0 for the maximum of viscosity of caseinogen in alkaline solution. Owing to the

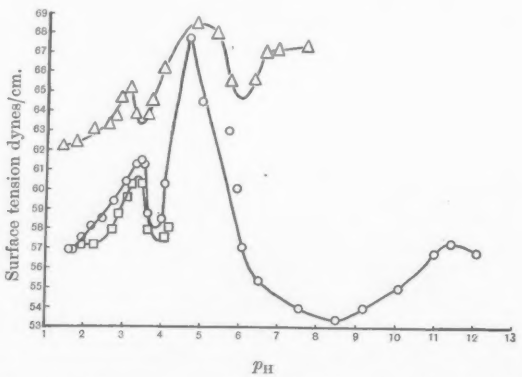

Fig. 1. Effect of p_H on the surface tension of caseinogen and egg-albumin solutions.

○ 1 % caseinogen in presence of HCl and NaOH
□ ,, ,, ,, H_3PO_4
△ 0·3 % egg-albumin ,, HCl and NaOH

limited solubility above p_H 3·0 it would not be expected that the maxima of viscosity and osmotic pressure of caseinogen solutions would agree with the minimum surface tension, since the degree of solubility greatly influences the properties, in fact Loeb [1922, 1] states that the maximum for the influence of caseinogen hydrochloride on viscosity lies at p_H equal to or greater than 3·0. The surface tension curve seems to indicate that the Donnan equilibrium gives a maximum potential difference at a higher p_H than 3·0, since the minimum surface tension is at p_H 3·8–3·9. If solubility were the only factor affecting the surface tension no minimum would be observed.

On the alkaline side the fall of surface tension was much greater, the minimal value being at p_H 8·5, followed by a rise in surface tension with increasing alkalinity with a possible secondary maximum at about p_H 11·5.

The effect of p_H on the surface tension of egg-albumin solutions.

A similar series of determinations of surface tension at various hydrogen ion concentrations within the range of the quinhydrone electrode was carried out with crystallised egg-albumin solutions. The stock solution of albumin purified as described above was mixed with the requisite quantity of HCl or NaOH to give a final concentration of 0·3 % albumin. The solutions were kept for 24 hours at 25° before determining the surface tension at 25°. The results are also shown in Fig. 1, where it will be seen that the maximum surface tension of egg-albumin solutions is also at the isoelectric point, p_H 4·8. Like caseinogen, it does not attain the value for pure water as flocculated isoelectric gelatin does, which is as expected from the solubility of isoelectric albumin. Loeb [1922, 2; 1923] has also shown that neutral salts even in very low concentrations may impart a surface potential to isoelectric denatured albumin, and experiments on the effect of neutral salts on protein solutions to be described later also show that in low concentrations they decrease the surface tension; hence the presence of ammonium sulphate even in concentration of $M/3000$ will tend to lower the surface tension of isoelectric albumin solution, accounting for the value of 68·5 dynes/cm. instead of the theoretical value 72·2 dynes/cm. of pure water.

A sharp secondary maximum of surface tension on the acid side of the isoelectric point occurs at p_H 3·0. The minimum between these maxima is at p_H 3·4–3·5; thus the maximum lowering of surface tension agrees well with Loeb's values of p_H 3·4 for the maximum osmotic pressure and p_H 3·6 for the maximum membrane potential.

So far as the alkaline side has been investigated there is a minimum surface tension at p_H 6·0. Loeb found the potential difference of albumin to be still increasing at p_H 5·8 from the minimum value at the isoelectric point.

As discussed in an earlier communication [St Johnston and Peard, 1925], the surface tension of a protein solution should be a maximum when the potential difference is a minimum and *vice versa*, thus, as in the cases of gelatin and caseinogen, the fall of surface tension of egg-albumin solutions is explicable up to a point by Donnan's membrane equilibrium theory.

The probable cause of the further fall in surface tension in more acid solutions, observed in all the proteins investigated, giving rise to the second maximum of surface tension, which is not accountable by the Donnan equilibrium will be dealt with in a succeeding section.

The effect of neutral salts on the surface tension of caseinogen solutions.

Since caseinogen is only completely soluble in HCl between about p_H 1·6 and 3·0, a solution of 1 % caseinogen in $N/100$ HCl of original p_H 2·46 was used to study the effect of neutral salts on the surface tension in acid solution. Since on the acid side of the isoelectric point the influence of the anion is greatest, all the salts investigated had the same cation, sodium, to eliminate any possible effect due to the cation. The salt to be added was weighed out and dissolved in the solution at the same time as the caseinogen, keeping the solution for 24 hours at 25° as usual. The effect of $NaNO_3$, NaCl and Na_2SO_4 and also of a non-electrolyte, glucose, is shown in Fig. 2. All the salts, in spite of a difference in valency of the anion, produce the same lowering of surface tension below that of the original caseinogen solution up to a concentration of $M/256$, and in greater concentrations produce a rise in surface tension, which is greater for Na_2SO_4 than for $NaNO_3$ and NaCl. The effect of glucose on the surface tension was practically negligible.

In experiments on the influence of neutral salts on protein solutions, the effect of the neutral salts on the p_H must always be considered. In all cases the p_H as determined electrically was increased slightly by the addition of a salt, in proportion to the concentration added, as was observed by Chick and Martin [1911]. NaCl and $NaNO_3$ at the same concentration raised the p_H to approximately the same extent, whilst Na_2SO_4 caused about double the rise due to NaCl or $NaNO_3$ at the same concentration. The rises of p_H were, however, not enough to account for the changes in surface tension observed. At p_H 2·46 it will be seen from Fig. 1 that an increase in p_H should raise the surface tension; up to $M/256$ for all three salts a fall of 2 dynes was observed, whereas the p_H changed from 2·46 to 2·52 for NaCl and $NaNO_3$ and to 2·66 for Na_2SO_4, which should have raised the tension by 0·2 and 0·6 dyne respectively.

At $M/32$ the surface tension in presence of NaCl and $NaNO_3$ had risen again so that it was only 1 dyne below the original value, whilst the p_H had increased to 2·64, a rise of p_H 0·18, which should increase the surface tension by 0·5 dyne. Na_2SO_4 at $M/32$ raised the surface tension 3 dynes above the original value, with an increase of p_H 0·36, which alone would only increase the surface tension by 1 dyne. Thus the changes in surface tension observed in presence of neutral salts are far greater than can be accounted for by the changes in p_H produced.

If the surface tension of protein solutions depended only on the Donnan effect at the p_H of the above experiment, addition of neutral salts even in lower concentrations than $M/1024$ should depress the potential difference, as shown by Loeb [1922, 1], and therefore raise the surface tension, and the effect should be in proportion to the valency of the anion added. Evidently this is not what takes place in concentrations below $M/256$ since the sulphate ion has exactly the same effect as the chloride and nitrate ions in lowering

the surface tension. A possible cause of the fall of surface tension in presence of neutral salts below a concentration of $M/256$ is that denaturation of the protein is increased by their presence. Mastin and Schryver [1926] have shown that denaturation of egg-albumin occurs on standing in presence of acid and neutral salt, and in a succeeding section it will be shown that denaturation produces a lowering of surface tension and that denaturation is occurring during the 24 hours at 25° in solutions of caseinogen at p_H 2·46 or of albumin at p_H 2·6. This may be increased by the presence of salts thus lowering the surface tension, though in greater concentration than $M/256$ the Donnan effect and the lyotropic effect of removal of associated water predominate and raise the surface tension, so that when the salt concentration is sufficiently great the protein is salted out completely and the surface tension becomes that of the salt solution.

The effect of a neutral salt on the surface tension of albumin solutions.

The effect of Na_2SO_4 upon 0·3 % albumin solution in presence of HCl was followed in the same manner as for caseinogen, and found to produce the same results. The p_H of the solutions was originally 2·6, which is at a similar point to a caseinogen solution of p_H 2·46 in respect to the surface tension-p_H curves of Fig. 1. The p_H rose with the addition of Na_2SO_4 up to a value of p_H 3·0 when in presence of M Na_2SO_4.

No sign of precipitation occurred up to a concentration of $M/16$ Na_2SO_4, where there was slight turbidity. With $M/4$ Na_2SO_4 there was much precipitation and with M Na_2SO_4 (nearly saturated solution) the albumin was salted out almost entirely.

The effect on the surface tension is also shown in Fig. 2, where it will be seen that there is a similar fall of surface tension to that of caseinogen solutions to a minimum at $M/256$, followed by a rise. After $M/16$, however, the rise is much increased, when the albumin is almost entirely salted out and the surface tension is nearly that of water. Evidently in the stronger solutions the lyotropic effect of the salt in withdrawing associated water from the protein is acting as well as the effect due to the salt on the Donnan equilibrium.

The denaturation of egg-albumin solutions in presence of acids.

In a previous communication [St Johnston and Peard, 1926] it was suggested that the fall in surface tension of gelatin solutions acid to the maximum of surface tension at p_H 2·0–2·2 might be due to hydrolysis or denaturation of the protein. No evidence of immediate hydrolysis as shown by formaldehyde titration was found however, though on standing for some months the most acid solutions had increased in formaldehyde titre and the surface tension had fallen further. Harris [1923], also from formaldehyde titrations on fresh and denatured egg-albumin, came to the conclusion that no detectable change occurred on denaturation, although there is a change

in the sulphur linkages, and Lewis [1927] found no detectable change in titratable acidic or basic groups of haemoglobin on denaturation. Young [1922] found that light-denatured albumin had a lower surface tension than before exposure.

Since caseinogen and egg-albumin behave similarly to gelatin in respect to the effect of p_H on the surface tension, experiments were undertaken with egg-albumin to ascertain if the fall in surface tension in the more acid solutions, which is not in accordance with the membrane equilibrium theory, could be ascribed definitely to denaturation. Whatever the nature of the change in the protein on denaturation, it is possible that it might lower the surface tension.

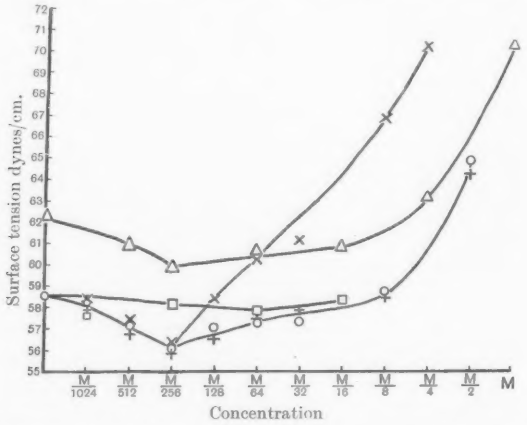

Fig. 2. Effect of neutral salts on the surface tension of caseinogen and egg-albumin solutions.

○	1 % caseinogen	in presence of	$NaNO_3$	and	HCl originally	p_H 2·46
+	,,	,,	NaCl	,,	,,	p_H 2·46
×	,,	,,	Na_2SO_4	,,	,,	p_H 2·46
□	,,	,,	glucose	,,	,,	p_H 2·46
△	0·3 % egg-albumin	,,	Na_2SO_4	,,	,,	p_H 2·60

Egg-albumin, free from salts, is not coagulated by heating, whilst denaturation is greatly accelerated by heating; thus a dialysed albumin solution is very suitable for studying the effect of denaturation on surface tension, since if coagulation occurred there would inevitably be a rise of surface tension to that of pure water on complete coagulation. The solutions employed were of such a salt concentration that only those between the isoelectric point and neutrality were coagulable. As will be seen from Fig. 1, the surface tension of albumin solutions varies with the p_H in a manner explicable by Donnan's membrane equilibrium theory between p_H 3·0 and neutrality, but below p_H 3·0 the surface tension again falls sharply with increasing acidity. If this further fall is due to denaturation occurring while standing for 24 hours at 25° in presence of acid giving a p_H less than 3·0, the effect should become more marked on accelerating the denaturation by heating.

Sørensen [1925] has shown that coagulation proceeds most rapidly near the isoelectric point, whilst the preceding process of denaturation occurs more rapidly the greater the hydrogen ion concentration. Somewhat similar experiments were therefore carried out and the surface tensions observed. A recently dialysed, clear solution of albumin (B) was used, and 0·3 % solutions of various p_H were kept at 25° for 24 hours. The salt content was about $M/1500$ $(NH_4)_2SO_4$ which was insufficient to cause coagulation of the denatured protein on heating except in the region of the isoelectric point. The surface tension was determined at 25°, then the solution, still in the surface tension bubbler, was put in a water-bath at 60° for exactly 15 minutes, quickly cooled under the tap and the surface tension redetermined at 25°.

Chick and Martin [1911, 1912] have shown a fixation of acid on denaturation of egg-albumin. It was therefore necessary to determine the changes in p_H occurring under the conditions of the experiment. Another preparation of dialysed albumin was used for this purpose and the p_H of the 0·3 % solutions determined after they had stood 24 hours at 25° and again after heating for 15 minutes at 60°. It will be seen from Table II that the only appreciable changes in p_H occur between p_H 3·8 and p_H 2·5, which show a maximum increase of p_H 0·15; this would have some effect on the surface tension, but it will be seen from Table III that over the range of p_H 3·0–4·0 there is practically no change of surface tension. From Fig. 1 it will be seen that a rise of p_H between 2·5 and 3·0 would tend to raise the surface tension, whereas from Table III at p_H 2·85 it falls. Between p_H 3·0 and 3·5 increase of p_H would lower the surface tension and between 3·5 and the isoelectric point raise it, only at p_H 4·0 does this begin to occur, when coagulation is also beginning. Thus the change of p_H on denaturation by no means accounts for the changes of surface tension observed, and where the maximum fall of surface tension is observed no change of p_H occurs. That no change occurs on further heating the most acid solutions seems to be due probably to the fact that a certain amount of denaturation and consequent fixation of acid has taken place while standing for 24 hours at 25°.

Table II.

Original p_H	p_H after heating 15 mins. at 60°	Change of p_H
6·63	6·64	+·01
3·78	3·93	+·15
3·33	3·47	+·14
2·78	2·91	+·13
2·54	2·62	+·08
2·38	2·39	+·01
2·24	2·25	+·01
1·87	1·87	0
1·70	1·71	+·01

The surface tension results are recorded in Table III.

Thus in solutions alkaline to and including the isoelectric point a heavy coagulation accompanied by a rise in surface tension occurred, and at the isoelectric point the surface tension rose to that of water.

Table III.

p_H	Surface tension: Before heating (dynes per cm.)	Surface tension: After 15 mins. at 60° (dynes per cm.)	Change of surface tension (dynes per cm.)	Appearance after heating
6·76	67·9	70·2	+2·3	Coagulation
6·28	65·5	68·6	+3·1	,,
5·32	68·0	71·3	+3·3	,,
4·77	68·5	72·1	+3·6	,,
4·00	65·9	68·0	+2·1	Slight turbidity
3·66	64·0	64·0	0	Clear
3·22	63·8	63·9	+0·1	,,
3·10	64·7	64·6	−0·1	,,
2·85	63·7	63·1	−0·6	,,
2·03	63·3	62·5	−0·8	,,
1·79	63·3	62·2	−1·1	,,

The solution of p_H 4·0 showed a marked rise in surface tension with only a very slight turbidity, whilst solutions between p_H 3·0 and 4·0 gave no change of surface tension or of appearance.

From the point at which the solutions became acid to the maximum of surface tension at p_H 3·0 a marked fall in surface tension occurred on heating, the fall increasing with increasing hydrogen ion concentration.

The experiment was carried a stage further by heating the solutions to 60° for 15 minutes, then bringing to the isoelectric point by the addition of a buffer solution and again heating. In this case an albumin solution (A) which had been prepared about 6 weeks previously was used. This solution, again of 0·6 % concentration but with only about $M/3000$ $(NH_4)_2SO_4$ and saturated with toluene, had been kept at about 4° in the dark and had become opalescent with a slight deposit, agreeing with the observation of Sørensen [1926] who found a similar change under the same conditions, namely, a slight deposit of denatured albumin, whilst the filtered solution could be recrystallised, giving material with normal properties, except that it contained less phosphorus than fresh albumin. The solution was filtered and solutions of various p_H prepared and kept at 25° for 24 hours as before. The surface tensions were found to be lower than the values previously determined for the same p_H; thus some change had occurred, but this was immaterial to the experiment.

The results are given in Table IV.

Table IV.

(*a*) Experiments as in Table III.

p_H	Surface tension: Before heating (dynes per cm.)	Surface tension: After 15 mins. at 60° (dynes per cm.)	Change of surface tension (dynes per cm.)	Appearance after heating
4·9	64·5	69·4	+4·9	Coagulation
4·0	62·2	65·2	+3·0	Slight turbidity
2·6	62·1	61·0	−1·1	Clear
1·43	61·4	60·1	−1·3	,,

(*b*) Equal volume 0·2 *N* acetate buffer, p_H 4·87, added to above heated solutions.

Original p_H	p_H after buffer added	Surface tension after: Buffer added (dynes per cm.)	Heating 15 mins. at 60° + buffer (dynes per cm.)	Change of surface tension (dynes per cm.)	Appearance after heating
4·9	4·81	69·5	69·8	+0·3	Coagulation still present
4·00	4·88	67·5	67·6	+0·1	Slightly increased turbidity
2·60	4·81	64·0	67·8	+3·8	Ditto.
1·43	4·56	64·8	66·4	+1·6	Ditto.

(*c*) Control experiment: 0·2 *N* acetate buffer, p_H 4·87, added to solutions as above not previously heated at 60°.

Original p_H	p_H after buffer added	Surface tension after: Buffer added (dynes per cm.)	Heating 15 mins. at 60° (dynes per cm.)	Change of surface tension (dynes per cm.)	Appearance after heating
4·9	4·81	66·5	66·5	0	No coagulation
4·00	4·88	66·4	66·9	+0·5	,,
2·60	4·81	64·8	65·4	+0·6	,,
1·43	4·56	65·2	65·6	+0·4	,,

The effect of heating for 15 minutes at 60° was the same as before, and on the addition of an equal volume of an 0·2 *N* acetate buffer, p_H 4·9, and again heating for 15 minutes at 60°, rises of surface tension were now observed in those solutions which had previously been acid to p_H 3·0 but not in the others. There was no coagulation after adding the buffer, though for surface tension determinations this was an advantage. Control experiments were made by adding the buffer to equal volumes of solutions which had not been heated at 60°; these were thus only heated at that temperature in the neighbourhood of the isoelectric point. It was observed that the presence of the buffer prevented the coagulation of the nearly isoelectric albumin, which coagulated readily when heated alone. Lepeschkin [1922] found that some salts have an accelerating and others a retarding effect on the coagulation-velocity of denatured albumin, whilst Lewis [1926] found the denaturation-velocity accelerated or retarded according to the salt and its concentration. Apparently with the relative concentrations present here, namely 0·15 % albumin and 0·1 *N* acetate, there is a retardation sufficient to prevent coagulation during 15 minutes at 60°.

The control experiment, Table IV (*c*), shows that a slight amount of denaturation during the 24 hours at 25° has occurred in all but the isoelectric solution, as, although no visible coagulation occurred, the surface tension rose slightly on heating in presence of the buffer. The solution originally most acid was not brought quite to the isoelectric point by the addition of the buffer, hence the rise of surface tension was not as great as for the other two acid solutions, both in the control experiment and in experiment IV (*b*), although in experiment IV (*a*) it showed the greatest fall of surface tension after heating without buffer. Since denaturation is increased by heating, the falls of surface tension in acid solutions after heating recorded in Tables III

and IV (*a*) clearly indicate that denaturation is accompanied by a fall of surface tension. Coagulation is accompanied by a rise in surface tension as would be anticipated and is well shown by those solutions in Table III giving visible coagulation; thus in those solutions heated with buffer near the isoelectric point the rises of surface tension are due to coagulation of the previously denatured protein although it is insufficient to be visible. With the above-mentioned exception of the most acid solution, p_H 1·43, the greatest rises of surface tension on heating with buffer occur in those acid solutions which showed the greatest falls on previous heating without buffer. This further proves the falls in surface tension to be due to denaturation, since Sørensen [1925] has shown that the greatest amount of coagulation when brought to the isoelectric point occurs in those solutions which had previously been most denatured, namely, those with the greatest hydrogen ion concentrations originally.

The experiments bring out most clearly the marked increase in denaturation after p_H 3·0 which is the point of inflexion of the surface tension-p_H curve of albumin, hence the previous assumption that this was due to denaturation seems to be correct. Recent work by Mastin and Schryver [1926] has shown that denaturation of egg-albumin is brought about by long standing in the presence of acid and salt, and Chick and Martin [1911, 1912] and Lewis [1926] have proved the denaturation velocity to increase rapidly with the H or OH ion concentration from a minimum at neutrality. Hence there will be a certain amount of denaturation occurring in an egg-albumin solution during 24 hours at 25°, increasing with the acidity, and the above experiments show that a sudden great increase occurs after p_H 3·0. Since gelatin and caseinogen have similar surface tension-p_H curves to egg-albumin, with acid maxima at p_H 2·0 and p_H 3·4 respectively, it is reasonable to assume for these proteins also that denaturation is the cause of the fall of surface tension in solutions acid to these points.

Thus denaturation also accounts for the fall in surface tension of gelatin solutions on keeping over a long period, as reported previously [St Johnston and Peard, 1926], and for the low values obtained with albumin solution A given in Table IV compared with the values in Fig. 1 obtained some weeks previously.

Conclusion.

Although du Noüy has shown the surface tension phenomena of protein solutions to be due to the single molecules of proteins, the surface tension may be brought into line with other properties of colloidal behaviour as being dependent upon the Donnan equilibrium except where denaturation occurs or where adsorption or lyotropic effects of neutral salts predominate over the membrane equilibrium effect. As was remarked by Michaelis [1926], and would be expected since the charge on the particle is a minimum, the maximum surface tension is at the isoelectric point for the three proteins investigated, gelatin, caseinogen and egg-albumin, whilst the hydrogen ion concentrations

of the maximum lowerings of surface tension on either side of the isoelectric point agree fairly well with those of the maxima of osmotic pressure, viscosity and potential difference found by Loeb [1922, 1] for the same proteins.

The published results with other proteins are somewhat at variance, particularly in respect to the surface tension at the isoelectric point. Buglia [1908], Bottazzi [1912, 1913], Bottazzi and d'Agostino [1912, 1913] found the surface tension of proteins, in particular those of the body fluids, to vary with the acidity or alkalinity much as described above, though of course the p_H was not considered. Whereas in some cases suspensions of a serum-protein in water did not appreciably lower the surface tension, with other serum-proteins a minimum surface tension at the isoelectric point was apparently observed, and Bottazzi's inference that the undissociated protein produces the greatest lowering of surface tension has been generally quoted. Although Quagliariello [1921] found the same for haemoglobin, he considers it anomalous. Bottazzi's view was apparently confirmed for gelatin by de Caro [1925] but this was shown not to be so in a previous communication [St Johnston and Peard, 1926] and the maximum surface tensions of caseinogen and egg-albumin at their isoelectric points are a further confirmation that this is not the case.

The sensitiveness of surface tension determinations of protein solutions to slight differences of p_H, to denaturation and to small amounts of neutral salt seems a likely explanation of the variable results with other proteins, particularly as in most cases no account was taken of p_H.

Hence it appears that surface tension lowering is a general property of the proteins which varies with the hydrogen ion concentration, as do other properties dependent upon the Donnan equilibrium, but the influence of denaturation appears in more acid solution and in presence of neutral salts.

Summary.

1. The surface tension of caseinogen and egg-albumin solutions varies with the p_H similarly to that of gelatin solutions, being maximum at the isoelectric point and minimum at points corresponding to the maxima of other properties, whilst a secondary maximum occurs in more acid solutions.

2. Neutral salts in low concentration lower the surface tension of caseinogen and egg-albumin in acid solution, but raise it in a greater concentration.

3. Denaturation of egg-albumin lowers the surface tension, which accounts for the fall of surface tension in solutions acid to the secondary maximum in the surface tension-p_H curve, for the initial fall in presence of low concentrations of neutral salts, and for the fall in surface tension on long standing.

4. The surface tension of protein solutions appears to be a general property which can be brought into line with the other properties of proteins as being governed by the Donnan equilibrium when due allowance is made for denaturation and the lyotropic effect of neutral salts.

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CLXXIV. A NEW COLORIMETER BASED ON THE LOVIBOND COLOUR SYSTEM, AND ITS APPLICATION TO THE TESTING OF COD-LIVER OIL, AND OTHER PURPOSES.

By OTTO ROSENHEIM,

With an Addendum by EDGAR SCHUSTER.

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(*Received October 24th, 1927.*)

The need for the construction of an apparatus of the type described in this communication[1] arose during the course of work on the colorimetric estimation, by means of the arsenic—or antimony—trichloride reaction, of the chromogen contained in cod-liver oil and other fats, which is assumed to be identical with vitamin A [Rosenheim and Drummond, 1925; Carr and Price, 1926]. The difficulties which had to be overcome before this test could be applied quantitatively were twofold and were due (1) to the characteristic instability and transient nature of the brilliant blue colour developed in the test, and (2) to the variable tints of blue (*i.e.* greenish or reddish) produced and determined by the uncontrollable and variable admixture of yellow or red pigments in the oils to be tested. The formation of these adventitious colours excluded the employment of any one fixed standard for comparison, as well as the use of a colorimeter of the usual Duboscq type. Lovibond's Tintometer, on the other hand, enables a perfect matching of any mixture of colours to be made, but the apparatus in its present form is hardly suitable for the extremely rapid work essential in the above test. Considerable experience is required in the expeditious selection of the suitable standard glasses, which have to be inserted into the apparatus singly and exchanged by hand if unsuitable. This drawback has been overcome in the model illustrated (see Fig. 1) by arranging the standard glasses in frames containing ten each of the red, yellow and blue units, in such a way that they can be rapidly moved horizontally behind a window and below the cell (or test-tube) containing the coloured solution to be matched. A mounting of the standard glasses on discs was considered in the first instance. The horizontal arrangement, in preference to a vertical one, was chosen for technical reasons in discussion with Dr W. V. Thorpe, to whom I am also indebted for the construction of a small working model. The apparatus now described, which is entirely different in general arrangement and mechanical details from Dr Thorpe's model, was designed and constructed by Dr E. Schuster, who has kindly furnished the description of it given in this paper.

[1] The Tintometer, Ltd., Salisbury, have undertaken the manufacture of the apparatus, all rights in it having been put freely at their disposal.

The apparatus has proved itself not only eminently suitable for the purpose for which it was designed, but possesses certain advantages, which seem to justify a recommendation for its use in colorimetric work generally and in biochemistry in particular. Estimations made with it are free from the error inherent in those obtained by the use of Duboscq's colorimeter, or any of its modifications, in which two coloured solutions of different concentration are

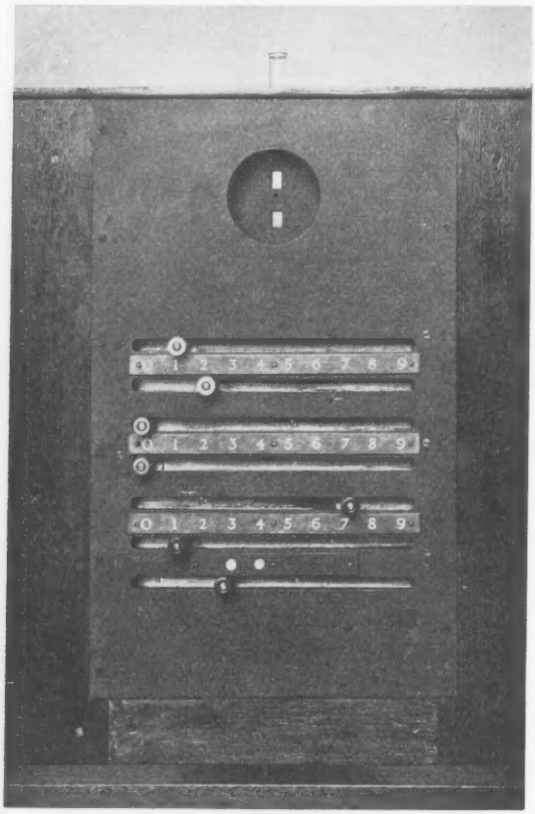

Fig. 1.

compared in layers of different thicknesses. This error, which may in extreme cases amount to as much as 25 %, is discussed fully by Stanford [1913, 1923]. Further, a well-known difficulty in biochemical colorimetric work, which cannot be overcome in the usual type of colorimeter, is due to the difference in tint between the colours given by the "unknown" and by the standard prepared from a pure substance respectively. The formation of adventitious

colours due to constituents, other than those to be determined, occurring in physiological fluids or extracts of organs is unavoidable. In the new apparatus the variable accidental colour admixtures can be easily matched, but only the essential colour value of the reaction itself is taken for the purpose of calculation (see example later). As a final advantage may be claimed the saving in time in preparing, as is usually necessary, a fresh standard solution for comparison. This is replaced by Lovibond's colour units, which are reproducible and permanent. The practical advantages of this colour system are recognised in industrial chemistry, where it has been used extensively since its introduction forty years ago [Lovibond, 1887], but references to its application are scanty in biochemical literature [Palmer and Kempster, 1919; Calvert, 1923; Baker and Marrian, 1927]. The accuracy of the colour units may be controlled spectrophotometrically [Gibson, Harris and Priest, 1927].

Although all the evidence so far available [Rosenheim and Webster, 1926, 1927; Willimott and Wokes, 1927; Accessory Food Factors Committee, 1927] is in favour of the specificity for vitamin A of the $AsCl_3$ (or $SbCl_3$) colour reaction, it seems advisable to await still further confirmation before recommending the colorimetric test for general use as a reliable substitute for the animal experiment. This caution seems to be the more necessary, in that the ease with which estimations can be carried out with the new colorimeter might encourage manufacturers, who have no facilities for controlling the results biologically, to rely on the colorimetric test exclusively.

In order to correlate the colorimetric with the biological test, it was decided after considerable experimental work to take 20 mg. of cod-liver oil as the standard amount for analysis. It was found that the daily administration of this quantity of a good average cod-liver oil, which served as a standard, was the minimum amount required to restore normal growth, *i.e.* to induce a weekly increase of 8–10 g. in rats kept on a diet free from vitamin A. The same amount of this oil equalled 10 blue standard units when mixed with 1 cc. of the reagent, and measured in a layer of 10 mm. thickness. As a preliminary indication, therefore, of the value of an unknown oil in vitamin A, it may be taken that 10 blue standard units correspond to one "rat unit" of vitamin A when the colorimetric comparison is made under the indicated conditions.

The following procedure, the outcome of two years' experience, is recommended for carrying out the test.

(*a*) *Arsenic chloride test.* The oil is measured by means of a Wright's capillary micro-pipette [Wright and Colebrook, 1921], graduated with mercury to hold 25 $mm.^3$ and delivering 20 mg. oil into a test-tube of clear white glass of 10 mm. internal diameter. 1 cc. of the reagent (pure $AsCl_3$) is delivered from a standard 10 cc. burette into the test-tube and a reading taken in the colorimeter immediately (time limit 30 seconds). The results are expressed in standard units of blue. Five consecutive tests are made in each case, differing on the average by $\pm$ 0·10 unit.

(*b*) *Antimony chloride test.* By means of a standard pipette 1 cc. of the oil is measured into a measuring flask of 5 cc. capacity. The pipette is rinsed out three times with chloroform, and the solution made up to volume with the same solvent. In order to make the results comparable with the $AsCl_3$ and the biological test, 0·2 cc. (= 40 mm.^{3} oil) of the solution is measured into a test-tube (10 mm. diameter) and mixed with 2 cc. of a 30 % antimony chloride solution in chloroform, delivered from a standard burette. Readings are taken immediately as above, but, owing to the slightly increased stability of the $SbCl_3$ reaction, a longer time limit is generally permissible, readings taken within 1 minute showing no greater difference than $\pm$ 0·10 unit as the average of five consecutive estimations. Results obtained on the same oils by different observers at different times agreed within $\pm$ 0·50 unit, showing the usual variations due to differences in colour perception of the observers.

Identical values are obtained by the use of either $AsCl_3$ or $SbCl_3$ as a reagent for the colour production. The latter reagent is conveniently prepared by dissolving the required amount of antimony trichloride (B.D.H.) in ordinary B.P. chloroform by rapidly warming to about 50° on a water-bath. A perfectly clear solution results, which keeps unchanged when stored in well-stoppered bottles. A stock solution, prepared 12 months ago and kept in a clear glass bottle under ordinary laboratory conditions, gave constant results when tested at intervals on the same oil. Since such a solution is more convenient in use than undiluted $AsCl_3$, it is preferable for general work, except in the rare cases where an extremely low result requires confirmation by the $AsCl_3$ reagent [Rosenheim and Webster, 1927; see also Wilson, 1927]. This lack of sensitiveness in the antimony reagent is clearly due to the dilution with chloroform of both the oil and the reagent. The explanation suggested by Wokes and Willimott [1927, 1] that a separation of the chloroform solution into two layers may have been the cause of the discrepancy observed is unwarranted, since such has never occurred in our experience when the solution is prepared and kept as above described. It might be pointed out in view of the attempts to increase the accuracy of the reaction [Wokes and Willimott, 1927, 2] that this need is not apparent, since the error of the colorimetric test is out of all proportion smaller than that of the biological animal experiment, which may be as much as 40 % with the technique at present in use.

Further applications. In applying the instrument to any of the colorimetric methods in use, it is only necessary to construct a reference curve by plotting on the ordinate the number of colour units, determined by the estimation of the colour value of solutions containing known amounts of the substance to be analysed, the latter being plotted on the abscissa. As an illustration may be given the curve (Fig. 2) obtained for cystine by the use of the phosphotungstic reagent of Folin and Looney [1922]. I am indebted to Mr W. K. Anslow for the construction of this curve.

The accuracy of the method is proved by the analysis of a solution containing 0·19 % cystine, the amount being unknown to the observer. The result

was 0·19 % by the colorimetric method and by calculation from the sulphur content.

It may further be suggested that the apparatus might be easily adapted in routine work to colorimetric p_H determinations, the colour values of various indicators expressed in Lovibond's standard units, covering a p_H range of 1·2–9·0, being available [Taub, 1927].

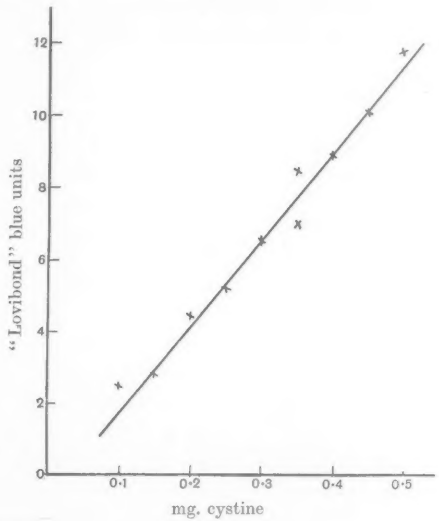

Fig. 2.

ADDENDUM. BY EDGAR SCHUSTER.

The apparatus consists of a wooden box, on the front of which is mounted a metal plate, painted a dead black. This plate with some of the surrounding woodwork is shown in the photograph (Fig. 1). Near its upper edge is a circular recess in which are two windows. A blackened tube with an eye-piece (not shown) fits this recess and serves to exclude extraneous light. The upper window is for viewing the coloured solution to be tested, and the lower one for the combination of coloured glasses with which its colour is matched. The liquid to be tested is contained in a standard test-tube (or cell) which is supported in a mounting so that its lower part comes between the upper window and the source of light. The top of the test-tube may be seen in the photograph. Behind the lower window are seven horizontal frames supported in a rack as closely as possible one behind the other. In the first frame are nine glasses, which, in combination with a blank space in the frame, provide a series of ten degrees of blueness differing by whole units: *i.e.* 0, 1, 2, 3, 4, 5, 6, 7, 8, 9. In the second frame the glasses form a scale of blueness rising by increments of one-tenth of a unit from 0 to 0·9. The third and fourth frames give units

and tenths of units respectively of yellow, and the fifth and sixth frames, of red. The seventh frame holds a blank space and three glasses, each having a tint of 10 units, and being blue, yellow and red respectively. Lateral movement of the frames is controlled by the seven knobs, which can be slid to and fro in the seven slots seen in the photograph between the circular recess and the bottom of the plate. The two upper knobs are coloured blue, and control the first and second frames. Between them is a scale, by which the tint of the glasses appearing behind the window may be read. As shown in the photograph the upper knob is opposite the 1 on this scale, and the lower knob, the 2. This means that the glasses shown are the 1 unit blue and the 0·2 unit blue, giving in combination 1·2 units of blueness. The next two knobs are coloured yellow and control the frames containing the yellow glasses, and the fifth and sixth knobs are coloured red and operate the red glasses. Scales similar to that between the two blue knobs are fitted between the two yellow and the two red. The lowest knob is black and moves the seventh frame; whether one of the three glasses or the blank space is showing is indicated by the row of coloured discs—white, blue, yellow or red which are fixed above the slot.

The mechanical arrangement by which the knobs control the movement of the frames is as follows: each knob is screwed to the middle of a long strip of steel, lying in the inside of the apparatus and supported horizontally by two guides carried on vertical steel bars. A descending member of the correct length is fixed to each end of each frame and its lower end is connected with the corresponding horizontal strip by a suitable distance piece. It is obvious that the number of frames and of standard glasses carried in each may be modified to suit special requirements.

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CLXXV. NOTE ON THE INDUCED FLUORESCENCE OF ERGOSTEROL.

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(*Received November 3rd, 1927.*)

A BRILLIANT blue fluorescence is observed when ergosterol is exposed to a source of ultra-violet rays (mercury vapour lamp) from which the visible rays are screened off by "Wood's glass" and a screen of bromine vapour. Eight specimens of different origin were examined, four of which had been prepared from ergot and four from yeast. Under the same conditions pure cholesterol, freed from ergosterol by bromine, showed no fluorescence. The phenomenon stands in no relationship, however, to the formation of vitamin D from ergosterol by ultra-violet irradiation. It has been found that under certain conditions preparations of non-fluorescent ergosterol can be obtained, which are rendered as highly antirachitic by irradiation as the fluorescent ones. Moreover, a specimen of cholesterol which could not be activated and was free from ergosterol, as shown by the spectroscopic test, possessed a strong blue fluorescence. This was traced to a previous treatment with charcoal (norite) during recrystallisation from alcohol, and a similar treatment induced fluorescence in non-fluorescent ergosterol. Since the same result is also produced by repeatedly recrystallising non-fluorescent ergosterol from ordinary ether, kept in light and containing peroxides, the conclusion seems justified that the fluorescence of sterols under the above conditions is due to the formation of traces of oxidation products.

CLXXVI. THE PHYSIOLOGICAL RÔLE OF VITAMIN B.

PART V. THE RELATION OF INANITION TO VITAMIN B DEFICIENCY IN PIGEONS.

By GUY FREDERIC MARRIAN, LESLIE CHARLES BAKER, JACK CECIL DRUMMOND and HERBERT WOOLLARD.

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(*Received October 26th, 1927.*)

Prefatory Note.

Throughout this paper vitamin B refers to the accessory food factors present in yeast. We have satisfied ourselves that pigeons fed on a synthetic diet of the type used throughout this work but supplemented with 4 % of yeast extract that has been heated with alkali for 4 hours to destroy the "anti-neuritic" factor behave exactly as do those which receive the unsupplemented ration. The presence of the treated yeast extract, providing amongst other substances the heat-stable factor [Hassan and Drummond, 1927], does not ameliorate the condition, or retard the decline or the onset of nervous symptoms, at any rate in the case of birds feeding voluntarily.

Introduction.

In previous communications [Drummond and Marrian, 1926; Kon and Drummond, 1927] of this series it has been pointed out that a large proportion of the abnormalities exhibited by animals deprived of vitamin B is due to the inanition that results from the failure of appetite rather than to any specific effect of the deficiency itself.

From the studies of the rat and of the pigeon it appeared that the lowered body temperature and metabolism, the loss of weight and atrophy of most tissues, and the hyperglycaemia could be so regarded, but it was not certain from these studies or the researches of earlier workers how intimately the nervous manifestations and the hypertrophy of the adrenal glands were related to starvation.

The investigation reported in this paper was carried out with the object of throwing light on these two points.

Experimental.

In the first experiment the pigeons were divided into four groups in such a manner that the average weight of each group was as nearly as possible the same. All were deprived of food with the exception that ample supplies of water were available. It must be remembered that simple starvation may be considered as one form of vitamin B deficiency. The birds in groups A and C served to differentiate between the effects of withholding food in presence and absence of vitamin B, in that the latter received every day 10 cc. of an aqueous 10 % solution of yeast extract (marmite) administered directly into the crop by a rubber tube.

In addition there were two other groups, B and D, similar to A and C except that each bird received daily from 100 to 200 cc. of water introduced directly into the crop in two equal doses every morning and evening.

These two groups were studied in order to investigate the claims of Chamberlain, Bloombergh and Kilbourne [1911], Chamberlain and Vedder [1911] and of Eijkman and van Hoogenhuyze [1916] to have produced the characteristic nervous symptoms of vitamin B deficiency in fowls and pigeons by "washing out" the birds with large volumes of water during the course of starvation.

In the second experiment three groups of pigeons were fed on a diet deficient in vitamin B. An artificial diet of the type used by Randoin and Simonnet [1924] was used. The diet was composed as follows:

Rice starch	66 %	Butter	4 %
Caseinogen	16 %	Paper pulp	2 %
Agar-agar	8 %	Salt mixture (McCollum)	4 %

The birds in group F were allowed to feed voluntarily on the deficient diet. Those of group G were forcibly fed with about 20 g. of the diet daily, whereas those of group H were forcibly fed with about 15 g. of the deficient diet and in addition were given daily 1 g. of yeast extract, in order to serve as a control against the diet and the possible ill effects of the forcible feeding. The diet could be readily introduced into the crop in a coarsely powdered form by pouring it down a wide bore glass tube and regulating the rate of flow with a thin glass rod.

All the birds were starved for 24 hours before the "initial weight" was recorded, in order to eliminate errors due to a loaded crop.

General behaviour of the starving birds.

Apart from a steady loss of body weight and an initial fall in body temperature the birds did not display any striking symptoms for the first few days. Later the feathers became ruffled and the birds seemed disinclined for movement of any sort, although they appeared to be quite strong when they desired to walk or fly. The final phase usually appeared with great suddenness and was characterised by rapid onset of muscular weakness and a sharp fall

in the body temperature, closely resembling the condition described in rats [Drummond and Marrian, 1926]. This dramatic collapse nearly always occurred in the very early morning hours, and we think it probable that nervous symptoms similar to those which we observed in the birds of all groups have escaped the attention of most investigators in the past because close observation of the animals during the whole 24 hours was not maintained. Perhaps the most characteristic behaviour of the birds in the pre-mortal phase of inanition, whether vitamin B extracts were given or not, was a slow dropping forward of the head followed by a step forward to readjust the balance of the body. This movement would go on rhythmically for several hours, interrupted sometimes by retrograde steps and in certain cases by more or less well-marked head retraction and cartwheel turning. To this last point we will again refer.

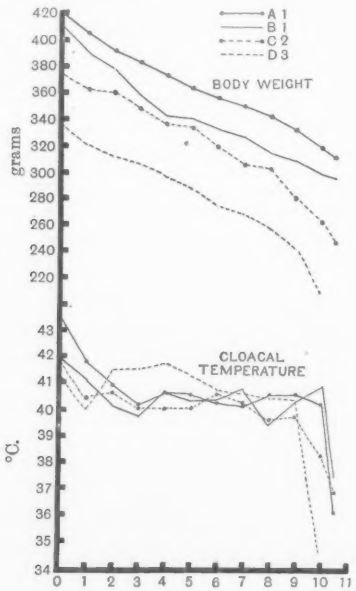

Fig. 1.

In Fig. 1 are drawn curves illustrating the weights and temperatures of birds in groups A–D. It will be seen that the loss of body weight is of the same order in all four groups, and that neither the rate of loss of body weight nor the course of the temperature curves is affected by the administration of amounts of yeast extract sufficient to supply the vitamin B requirements of the pigeon when feeding normally. These observations confirm in detail those that we have recorded for the rat.

General behaviour of the birds on the deficient diet.

For about the first week the birds in group F ate normally and the body weight was maintained. This period was followed by one showing progressively increasing fall in food intake, and a corresponding decrease in body weight. After about 30 days the birds usually showed the typical nervous symptoms of avian "beriberi," but in a few cases the pigeons failed to show any such symptoms and eventually died in a state of extreme weakness. In nearly all cases the birds by the time convulsions were observed had shown a loss of body weight of the same order as that shown by the starving birds, *i.e.* 30 to 40 % of the original weight.

The birds in group G behaved normally for about the first week, and appeared to be able to assimilate all the diet that was administered. Later, however, a small amount of the food was often vomited. A rough quantitative measure of the amount of rejected diet showed that at least 80 % of the diet fed was retained and digested. With no exception, the birds in this group developed violent convulsions within 20 days, at a period when the reduction of body weight was so slight as to be almost negligible.

In order to avoid errors in body weight due to a loaded crop, the food was removed quantitatively after the death of the bird, dried and the weight subtracted from the total weight of the bird before death.

Hypertrophy of the adrenal glands in inanition and vitamin B deficiency.

One of the most striking changes that have been observed in animals suffering from a deficiency of vitamin B is a well-marked hypertrophy of the adrenal glands [McCarrison, 1919, 1920; Kellaway, 1921; Findlay, 1921; Beznak, 1923; Lasowsky and Sumnitzki, 1926]. Changes of a similar order have also frequently been observed as a result of simple starvation [McCarrison, 1919; Vincent and Hollenberg, 1920, 1921; Findlay, 1921; Beznak, 1923]. It must, however, be borne in mind that complete deprivation of food also involves a deficiency of vitamin B, so that the fact that the adrenals hypertrophy as a result of starvation does not warrant the assumption that the enlargement is not specifically due to the deficiency of the accessory factors. Beznak [1923] indeed claims to have shown that no hypertrophy of the adrenal glands is exhibited by starving pigeons that are receiving ample supplies of vitamin B in the form of yeast extract.

In nearly all cases, the starving birds were killed during the final collapse which we have described previously. The vitamin B-deficient birds were killed immediately the first signs of convulsions appeared. Immediately after death the glands were dissected out and weighed. In a number of cases estimations of the adrenaline present were carried out by a modification of the Folin, Cannon and Denis method [Baker and Marrian, 1927]. Table I shows the results obtained from a number of normally fed pigeons.

Table I. *Normal pigeons starved 24 hours before experiment.*

Weight (g.)	Sex	Weight of adrenals (g.)	Weight of adrenals per kg. body weight	Adrenaline (mg.)	Adrenaline per 1 g. gland (mg.)	Adrenaline per kg. body weight (mg.)
372	♀	0·0195	0·0524	0·055	2·82	0·148
263	,,	0·0317	0·1206	0·106	3·34	0·403
331	,,	0·0196	0·0592	0·048	2·45	0·145
388	,,	0·0170	0·0438	0·045	2·65	0·116
334	,,	0·0191	0·0572	0·056	2·93	0·167
296	,,	0·0192	0·0648	0·044	2·29	0·148
326	,,	0·0270	0·0828	0·072	2·66	0·214
Average 330	—	0·0219	0·0687	0·061	2·73	0·191
320	♂	0·0244	0·0769	0·066	2·71	0·206
395	,,	0·0216	0·0547	0·067	3·08	0·169
309	,,	0·0270	0·0879	0·069	2·55	0·223
304	,,	0·0319	0·1049	—	—	—
386	,,	0·0211	0·0546	0·076	3·60	0·197
322	,,	0·0154	0·0478	0·052	3·37	0·161
344	,,	0·0302	0·0878	0·072	2·38	0·209
Average 340	—	0·0245	0·0735	0·067	2·95	0·194

It is evident from the figures in Table II that the adrenal gland is hypertrophied in the later stages of starvation and that this enlargement is not prevented by the administration of extracts rich in vitamin B. This is contrary to the conclusion reached by Beznak [1923], and we are unable to account for his results.

The birds in group F which were voluntarily feeding on the deficient diet also showed with one exception a definite but rather smaller hypertrophy (Table III). At first sight it might have appeared that this could be ascribed entirely to the complicating effects of inanition. However, the forcibly fed birds in group G, which only showed a very slight decrease in body weight, exhibited an enlargement of the glands of the same order as that shown by the birds in group F. The birds in group H showed no abnormalities of the adrenal glands. Both the weight of the glands and the adrenaline estimated were of the same order as for the normally fed pigeons.

There is considerable divergence of opinion as to whether the hypertrophy of the glands observed in vitamin B deficiency and starvation is medullary or cortical in origin. As a result of chemical determinations of the adrenaline content of the glands McCarrison [1920] is convinced that the hypertrophy is medullary. On the other hand Beznak [1923], adopting similar methods, could find no increase in the adrenaline content of the glands and concluded that the cortex alone hypertrophies. As a result of histological studies of the adrenals of pigeons fed on polished rice, Findlay [1921] and Lasowsky and Sumnitzki [1926] also concluded that the hypertrophy is cortical. Indeed Findlay claims to have shown that after curing a pigeon with yeast extract there is an immediate disappearance of a large amount of lipoid material from the cortex.

Our own results show that in all cases the total adrenaline present in the starving birds is considerably increased. In some instances this increase is proportional to the hypertrophy of the glands, from which it would seem

Table II.

Group	No. of bird	Sex	Initial weight (g.)	Final weight (g.)	Initial wt / Final wt	Weight of adrenals (g.)	Weight of adrenals per kg. body weight: Initial (g.)	Weight of adrenals per kg. body weight: Final (g.)	Total adrenaline present (mg.)	Adrenaline per 1 g. gland (mg.)	Adrenaline per kg. body weight: Initial (mg.)	Adrenaline per kg. body weight: Final (mg.)	Days starving	Remarks
A	38	♀	280	174	1·61	0·0388	0·1385	0·2229	—	—	—	—	11	Complete collapse
	35	,,	320	193	1·66	0·0374	0·1169	0·1938	0·108	2·88	0·337	0·559	12	
	2	,,	358	242	1·48	0·0275	0·0768	0·1136	—	—	—	—	10½	Quite strong and steady
	3	,,	330	215	1·54	0·0602	0·1824	0·2800	—	—	—	—	10½	Complete collapse. Slight head retraction just before death
	4	,,	325	228	1·42	0·0457	0·1407	0·2005	—	—	—	—	7½	Not very weak. Loss of equilibrium after swinging
	5	,,	295	191	1·54	0·0329	0·1115	0·1722	—	—	—	—	10	Cartwheels and head retraction
Average		♀	318	207	1·54	0·0404	0·1278	0·1972	—	—	—	—		
	33	♂	380	226	1·68	0·0530	0·1394	0·2345	0·117	2·21	0·308	0·518	13½	Very weak. Slight head retraction just before death
	1	,,	405	310	1·31	0·0274	0·0676	0·0884	—	—	—	—	10½	Quite strong and steady
	6	,,	328	210	1·56	0·0610	0·1859	0·2905	—	—	—	—	8½	Loss of equilibrium after swinging
Average		♂	371	249	1·52	0·0471	0·1310	0·2045	—	—	—	—		
B	3	♀	323	220	1·47	0·0193	0·0590	0·0880	—	—	—	—	10½	Quite strong and steady
	1	♂	390	295	1·32	0·0455	0·1166	0·1542	—	—	—	—	10½	Quite strong and steady
	2	,,	304	253	1·44	0·0360	0·0989	0·1423	—	—	—	—	9½	Very weak. Cartwheels and head retraction
	4	,,	330	227	1·45	0·0384	0·1164	0·1691	—	—	—	—	8	Complete collapse
	6	,,	275	199	1·38	0·0530	0·1927	0·2660	—	—	—	—	6	Loss of equilibrium after swinging and head retraction
Average		♂	340	244	1·39	0·0432	0·1312	0·1829	—	—	—	—		
C	42	♀	310	208	1·49	0·0320	0·1032	0·1490	0·100	3·12	0·322	0·480	11	Very weak
	43	,,	330	211	1·56	0·0451	0·1366	0·2157	0·096	2·13	0·291	0·455	17	Weak
	1	,,	315	205	1·54	0·0448	0·1422	0·2185	—	—	—	—	7½	Loss of equilibrium after swinging
	4	,,	352	247	1·43	0·0480	0·1362	0·1943	—	—	—	—	10½	Disturbed by swinging. Head retraction and convulsions just before death
	5	,,	323	229	1·41	0·0512	0·1585	0·2236	—	—	—	—	9½	Very weak. Head retraction for short periods
	6	,,	286	200	1·43	0·0442	0·1545	0·2210	—	—	—	—	6½	Loss of equilibrium after swinging
Average		♀	319	217	1·47	0·0442	0·1385	0·2037	0·098	2·63	0·307	0·467		
	41	♂	340	210	1·62	0·0692	0·2353	0·3295	0·107	1·84	0·315	0·509	13	Marked head retraction. A tendency to turn cartwheels
	40	,,	330	207	1·59	0·0436	0·1321	0·2106	0·104	2·39	0·315	0·502	14½	Very weak
	39	,,	360	213	1·69	0·0577	0·1603	0·2709	0·099	1·72	0·275	0·465	15	
	2	,,	363	246	1·47	0·0428	0·1179	0·1739	—	—	—	—	10½	Quite strong and steady
Average		♂	348	219	1·59	0·0533	0·1614	0·2462	0·103	1·88	0·302	0·492		
D	3	♀	320	213	1·50	0·0444	0·1387	0·2084	—	—	—	—	10	Very weak
	6	,,	291	210	1·39	0·0449	0·1543	0·2138	—	—	—	—	8	Very weak. Slight tendency to head retraction
Average		♀	306	212	1·44	0·0447	0·1465	0·2111	—	—	—	—		
	1	♂	375	274	1·36	0·0479	0·1277	0·1748	—	—	—	—	7½	Cartwheels and head retraction
	2	,,	351	276	1·27	0·1144	0·3259	0·4144	—	—	—	—	7	Very weak. Slight tendency to head retraction
	4	,,	341	242	1·41	0·0334	0·0983	0·1384	—	—	—	—	9	Very weak
	5	,,	290	209	1·39	0·0552	0·1903	0·2641	—	—	—	—	6	Very weak
Average		♂	339	250	1·36	0·0628	0·1856	0·2479	—	—	—	—		

reasonable to suppose that in such cases the hypertrophy is medullary. In other cases, however, low figures for the adrenaline per g. of the gland were obtained although the total adrenaline present was above normal. This may either represent an approach to exhaustion of the adrenaline reserves of the gland in the last stages of inanition, or else an additional hypertrophy of the cortex.

The results obtained on the forcibly fed pigeons are of considerable interest, since in every case the total adrenaline present was either normal or slightly low, and in every case the adrenaline per g. of gland was definitely low (Table III). Since all these birds were killed at the first sign of convulsive symptoms, and at a time when they were muscularly strong, it does not seem possible that an exhaustion of the adrenaline in the gland had occurred. A possible explanation of these results would be to suppose that in "beriberi" uncomplicated by the effects of inanition only the cortex undergoes hypertrophy, and that in starvation, and "beriberi" associated with starvation, the hypertrophy is mainly medullary, but no decision can be reached without further experiments.

Blood-sugar of pigeons during inanition.

It is a clearly established fact that pigeons showing typical symptoms of "beriberi" may often show a distinct hyperglycaemia [Funk and Schönborn, 1914; Funk, 1920; Randoin and Lelesz, 1925; Collazo, 1923]. Kon and Drummond [1927] carefully controlled a series of pigeons against the complicating factors of starvation by feeding a second group of birds with the same amounts of food as were being voluntarily consumed by those receiving the deficient diet, at the same time administering excess of vitamin B in the form of yeast extract. The blood-sugars of these two groups were identical and they concluded that the hyperglycaemia observed during vitamin B deficiency is wholly attributable to inanition.

Blood-sugar determinations carried out on samples removed from the wing vein on our own two groups of starving pigeons (A and C) tend to support this conclusion (Table IV) and clearly show that the feeding of yeast extract during starvation had no effect on the blood-sugar. Except in the last stages of inanition both groups show a slight but distinct hyperglycaemia compared with the control group of normally fed birds.

The normal control birds were starved for 24 hours previous to the determinations in order that the blood-sugar should fall to the resting level.

A series of estimations of blood-sugar made during the course of the experiment shows that the slight hyperglycaemia which appears after the first day or two of deprivation of food and which persists for several days is followed by a marked hypoglycaemia (Fig. 2). The fall in the concentration of blood-sugar occurs very suddenly and is contemporary with the fall of temperature which characterises the pre-mortal phase. This picture is similar in all respects to that we have described in the case of the rat [Drummond and Marrian, 1926].

Table III.

Group	No. of bird	Sex	Initial weight (g.)	Final weight (g.)	Initial wt / Final wt	Weight o adrenals (g.)	Weight of adrenals per kg. body weight: Initial (g.)	Weight of adrenals per kg. body weight: Final (g.)	Total adrenaline (mg.)	Adrenaline per 1 g. gland (mg.)	Adrenaline per kg. body weight: Initial (mg.)	Adrenaline per kg. body weight: Final (mg.)	No. of days on diet
F*	328	♀	303	208	1·45	0·0516	0·1703	0·2481	0·142	2·75	0·468	0·682	33
	329	♂	357	233	1·53	0·0414	0·1159	0·1776	0·093	2·25	0·261	0·399	26
	326	,,	349	250	1·39	0·0192	0·0550	0·0768	0·041	2·13	0·117	0·164	27
	39	,,	452	362	1·24	0·0446	0·0986	0·1232	0·075	1·68	0·166	0·207	29
	32	,,	387	286	1·35	0·0395	0·1020	0·1381	0·084	2·13	0·217	0·293	30
	46	,,	389	285	1·34	0·0434	0·1116	0·1523	0·139	3·20	0·357	0·488	34
Average		♂	387	283	1·37	0·0376	0·0972	0·1328	0·086	2·28	0·224	0·310	
G*	839	♀	301	245	1·23	0·0370	0·1229	0·1510	0·039	1·05	0·129	0·159	19
	3302	♂	411	406	1·01	0·0376	0·0915	0·0926	0·069	1·83	0·168	0·169	15
	718	,,	352	321	1·09	0·0546	0·1551	0·1701	0·072	1·32	0·204	0·224	13
	749	,,	394	390	1·01	0·0519	0·1317	0·1331	0·068	1·31	0·172	0·174	12
	25	,,	347	326	1·07	0·0400	0·1149	0·1227	0·038	0·95	0·109	0·117	16
Average		♂	376	361	1·05	0·0460	0·1233	0·1296	0·062	1·35	0·163	0·171	
H	994	♀	337	364	0·93	0·0284	0·0843	0·0780	0·062	2·18	0·184	0·170	17
	734	,,	332	357	0·93	0·0276	0·0831	0·0773	0·058	2·10	0·175	0·163	17
Average		♀	335	361	0·93	0·0280	0·0837	0·0777	0·060	2·14	0·180	0·167	
	872	♂	278	254	1·09	0·0260	0·0934	0·1024	0·070	2·69	0·252	0·275	17
	924	,,	317	323	0·98	0·0236	0·0744	0·0731	0·075	3·18	0·237	0·232	17
Average		♂	298	289	1·04	0·0248	0·0839	0·0878	0·073	2·94	0·245	0·254	

* All the pigeons in groups F and G showed typical convulsions.

We are inclined to attach importance to the fact that all birds showing head-retraction simulating "beriberi" convulsions were passing through this pre-mortal phase.

Table IV.

Normal. Blood-sugar %	Group A. Starving 4–9 days. Blood-sugar %	Group C. Starving 4–9 days. 1 g. yeast extract daily. Blood-sugar %
0·174	A 1 0·174	C 1 0·206
0·176	A 2 0·202	C 2 0·160
0·186	0·210	C 3 0·242
0·190	A 3 0·224	C 4 0·234
0·178	0·242	C 5 0·186
0·191	A 4 0·214	C 6 0·236
0·171	A 5 0·186	C 39 0·209
0·183	A 6 0·210	C 40 0·202
—	0·226	—
—	A 38 0·215	—
—	A 33 0·202	—
Average 0·181 %	0·209 %	0·209 %

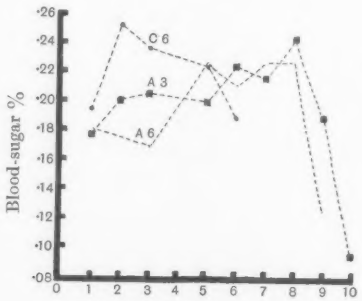

Fig. 2.

Head-retraction in pigeons during inanition.

Chamberlain, Bloombergh and Kilbourne [1911] and Chamberlain and Vedder [1911] reported that they were able to produce the typical nervous symptoms of "beriberi" in birds by "washing out" the tissues by administering large volumes of water during starvation. These results were confirmed by Eijkman and van Hoogenhuyze [1916] who showed that these symptoms could be cured by the administration of vitamin B notwithstanding starvation, and concluded that "polyneuritis" occurring as a result of feeding on a vitamin B-deficient diet cannot be caused by inanition.

It will be observed on referring to Table II that in each of the two groups A and B there were several cases of pigeons that had quite definite head-retraction. Pigeons A 5 and B 2 displayed extremely well-marked "polyneuritic" symptoms. In addition to showing a definite head-retraction, these birds turned "cartwheels"; their general appearance being indistinguishable

from that of those suffering from true vitamin B deficiency. In many other instances less marked but quite definite head-retraction was observed. By disturbing the equilibrium of all the birds these symptoms could be accentuated. It must be pointed out that these symptoms only occurred during the final pre-mortal phase, and that they were of considerably shorter duration than those observed in vitamin B deficiency.

The appearance of these typical "beriberi"-like symptoms in several of the birds in group A seems to indicate that flushing with water is an unnecessary procedure.

We were surprised to observe several cases of head-retraction in groups C and D which were receiving yeast extract daily. Pigeons C 4, C 5 and D 6, D 2 all showed slight but definite head-retraction for a short period before death, while C 41 and D 1 displayed more definite symptoms which were quite as well marked as any in groups A and B.

As these birds had been receiving yeast extract every day and had been given a dose within a few hours of the appearance of the convulsions, we have found it hard to reconcile our observations with those of Eijkman and van Hoogenhuyze that the convulsions exhibited by starving birds may be cured by administration of vitamin B[1].

Discussion.

Before we had carried out the experiments on the pigeons that received the food deficient in vitamin B forcibly we were convinced that the hypertrophy of the adrenals in ordinary vitamin B deficiency was due merely to inanition and was unrelated specifically to the absence of the vitamin. Obviously this view could be held no longer when enlarged glands were exhibited by the birds whose body weight had been more or less satisfactorily maintained by forcible administration of the food deficient in vitamin B. Moreover, the fact that the control birds, which received in addition to their ration of food a supplement providing an ample supply of vitamin B showed normal glands ruled out the possible explanation that hypertrophy might be caused by the actual process of forcible feeding.

As we have pointed out, our results could be explained on the rather improbable hypothesis that inanition produces medullary hypertrophy whilst deficiency of the factor B tends to cause enlargement of the cortical tissue.

We prefer to make no further comment at this stage of the enquiry on the results we have obtained. Further work is in progress which may throw light on the curious facts we have observed.

Regarding the appearance in the starving birds of convulsive seizures

[1] Dr R. R. Williams very kindly placed the manuscript of his paper [1927] in our hands whilst we were preparing the results of our work for publication. It is interesting that he noted that birds which exhibited nerve symptoms on diets containing ample supplies of vitamin B had lost from 30 to 40 % of their original body weight, a loss of the order of that shown by our starving birds at the time of onset of symptoms.

similar in general character to those exhibited by birds fed on diets deficient in vitamin B, it must be admitted that when the case of those which received a daily ration of yeast extract is considered the whole question becomes a much broader one than was originally raised by the experimental results of Chamberlain and his colleagues and Eijkman and van Hoogenhuyze.

In the first place we are not entirely satisfied that the symptoms shown by the birds "flushed" with water by Chamberlain were due to the washing out of vitamin B from the tissues as they suggested, because in our experience there was just as marked a tendency for the convulsions to occur in birds whose consumption of water was limited to the very small quantity they drank voluntarily. Secondly, we find it difficult to reconcile our observations with the statement of Eijkman and van Hoogenhuyze that they could cure "polyneuritis" in starving water-flushed birds with extracts of vitamin B.

We have described the onset of typical "beriberi" symptoms in starving pigeons which have been receiving daily an ample dose of vitamin B, and somewhat similar observations are recorded by Williams [1927] (see footnote to p. 1345).

The problem that faces us is not only to reconcile these conflicting views, but to provide an explanation of the curious fact that whereas pigeons feeding voluntarily on a ration deficient in vitamin B will usually show "beriberi" symptoms at about the 30th day, those forcibly fed on this diet do so at about the 16th day, and those deprived of food altogether at about the 9th.

We do not wish to commit ourselves to any definite opinion on these obviously difficult questions at present. For the time being we are, as we have already remarked, inclined to attach importance to the fact that whilst the nervous manifestations in the "beriberi" pigeon that is receiving food often appear at a time when the body temperature is not markedly subnormal and when the sugar concentration in the blood is usually slightly above the normal level, we have never observed the convulsions in starving birds except in the pre-mortal phase when the blood-sugar is very greatly reduced and the temperature is falling rapidly.

This difference raises in our minds the query whether the changes in the nervous system that are responsible for the convulsions may not be brought about by a variety of agents. This view is strengthened by reference to the work of Hess, which we have ourselves confirmed, and which showed that a condition closely simulating "beriberi" in pigeons can be produced by sublethal doses of cyanides. If this view be regarded as reasonable it attracts attention to the conception, many times expressed in the literature of experimental beriberi, that in vitamin B deficiency the underlying cause of the convulsions is a toxic substance, of which either the production is suppressed or the poisonous action is neutralised when vitamin B is administered. Dr Williams strikes the same note in the concluding paragraph of his discussion [1927, p. 1351].

In the investigations we are now reporting a considerable amount of

additional evidence was obtained from the histological examination of the peripheral nervous system of the pigeons. It all served to confirm the opinion previously expressed [Woollard, 1927] that the minor changes observed are attributable solely to inanition and are in no way directly related to vitamin B deficiency.

Summary.

(1) The administration of extracts rich in vitamin B does not prevent the hypertrophy of the adrenal glands in pigeons that are deprived of food. This is contrary to the conclusion of Beznak.

(2) In most cases the adrenaline content of the glands of starving pigeons, whether receiving vitamin B or not, is increased proportionately to the hypertrophy. This tends to confirm the opinion of McCarrison that the hypertrophy is medullary.

(3) A slight but definite hyperglycaemia is observed in pigeons during most of the course of inanition. It is followed by a marked fall in the amount of blood-sugar coincident with the pre-mortal fall in body temperature. The administration of extracts rich in vitamin B has no effect on the blood-sugar levels of such birds.

(4) Nervous symptoms similar to those observed in pigeons suffering from vitamin B deficiency were observed in a number of starving birds. The incidence of such symptoms was not increased by the daily administration of large volumes of water.

These convulsions cannot be due to a vitamin B deficiency resulting from starvation since several cases were observed in birds receiving daily administration of vitamin B.

(5) The symptoms observed in starving birds always occurred during the period just before death when body temperature and blood-sugar were much reduced. This is in contrast to the established fact that nervous manifestations in pigeons nourished on rations deficient in vitamin B usually appear when the temperature is but slightly reduced and the blood-sugar level is, if anything, above normal.

(6) By forcibly feeding pigeons on larger quantities of a diet deficient in vitamin B than they would voluntarily consume, typical nervous symptoms of beriberi were produced in about 16 days, at a time when the loss of body weight was almost negligible.

(7) The adrenal glands of the forcibly fed pigeons were hypertrophied to a considerable extent, although no corresponding increase of the adrenaline content was observed. At present no satisfactory explanation of this is forthcoming.

We wish to express our sincere gratitude to Prof. Bertrand for providing us with laboratory accommodation at the Pasteur Institute during part of this investigation.

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CLXXVII. A NOTE ON THE EFFECTS ON PIGEONS OF AN EXCLUSIVE DIET OF RICE MEAL, BRAN AND POLISH.

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Moore [1914] has described a condition in swine closely resembling beriberi, which, however, ensued from diets consisting largely of the outer coats of rice known as rice meal[1]. This product is, of course, rich in its content of anti-beriberi vitamin. This led the writer to test the effects on pigeons of exclusive diets of the several commercial by-products of rice milling. The products tested were rice bran, a coarse material consisting largely of the outer bran coats, rice meal, which is the material next removed from the grain in the milling process and rice polish, a fine relatively light coloured product containing a large percentage of starch as well as the last portions of the bran coats. A mixture of the products was also made in the proportion of 14 of bran to 10 of meal to 3 of polish, which is approximately the proportions in which the three products are obtained from the grain.

Exp. 1. It was desired first to prove the presence of adequate amounts of anti-beriberi vitamin in each of the several products mentioned above. Accordingly, four groups of four pigeons each were fed on polished rice *ad lib.*[2]. In addition each bird in one group was fed daily by hand 3 g. of rice polish; in another 3 g. of the meal; in the third the same quantity of bran, and in the fourth an equal amount of the mixture of the four products. The birds were weighed twice a week. All maintained their initial weights or made slight gains and continued to show a healthy and vigorous condition at the end of 60 days when the experiment was discontinued.

Exp. 2. A group of four pigeons was fed *ad lib.* on an exclusive diet of rice bran in the form of a stiff mash which was freshly prepared each day. No. 471 died after 32 days, having lost 49 % in weight; No. 472, at the end of 22 days with a loss of 42 % and No. 473 on the 22nd day having lost 41 %. The symptoms in these birds were not very characteristic, consisting of loss of appetite, a mild diarrhoea and steady decline in strength and weight. On examination of the sciatic nerves after staining by the Marchi method, a distinct fatty degeneration was readily detected in the fibres of all. No. 470

[1] Mr Moore in a private communication has verified this interpretation of the term rice meal and kindly submitted a small sample for inspection.

[2] Water was furnished from the Washington City water supply in all the feeding experiments described in this paper.

in the course of 40 days lost 26 % in weight, after which its weight remained stationary for an additional 80 days. At the end of this time the bird was killed, having exhibited no characteristic symptoms of disease. On examination, the fibres of the sciatic nerve were found to be extensively degenerated.

Exp. 3. An experiment precisely similar to the above was conducted, using rice meal instead of bran. All four birds lived for 120 days and, except for two instances of temporary decline, maintained their initial weights. At the end of this time, they were killed. The sciatic nerve fibres in all these birds appeared normal.

Exp. 4. Rice polish was fed under the same conditions to four pigeons. All four birds declined slightly during the second or third week but later recovered the loss in large part and lived throughout the 120 days of the experiment. No. 477 remained stationary at about 85 % of its initial weight throughout the latter half of the feeding period, but the remainder maintained approximately their initial weights and all were free from serious outward symptoms of disease. The fibres of the sciatic nerves of birds 475 and 476 showed no lesions, but those of 477 and 478 were found to be moderately degenerated.

Exp. 5. A mixture of 14 parts of bran, 10 of meal and 3 of polish was fed to four birds under similar conditions. No. 467 slowly declined to 64 % of its original weight and on the 65th day was found in a condition of complete paralysis, with severe neck retraction precisely similar in appearance to the condition of polyneuritis induced by feeding exclusively on white rice. Death followed on the same day. No. 468 declined to 76 % of its initial weight but lived for 120 days at this lower level without other evidence of disease. Nos. 466 and 469 retained weight and strength throughout the feeding period. After 120 days the birds remaining were killed as in the previous experiment. The sciatic nerve fibres of No. 467 were slightly degenerated but those of the remainder betrayed nothing abnormal.

Exp. 6. Six pigeons were fed in the same manner on another sample of commercial rice polish which, however, was identical with the first in appearance. These birds all declined fairly rapidly, losing from 30 to 40 % in weight. Nos. 328 and 329 died in 23 days; No. 330 in 10 days; No. 331 in 36 days; No. 332 in 13 days, and No. 333 in 15 days. No. 328 showed a slight ataxia and 333 a moderate neck retraction on the day previous to death. Degeneration of the sciatic nerve fibres was fairly pronounced in 328; slight in the remainder, being especially slight in 333.

It seems evident from the above experiments that rice mill by-products are distinctly harmful to pigeons when fed as the exclusive diet. Apparently there is a great variation in the harmfulness of different samples, an observation which is in entire accord with that of Moore as to the effects of rice meal on swine. It also seems likely that pigeons are more resistant than swine to the harmful factor, although there appear to be extremely wide individual variations in pigeons in this respect.

McCollum, Simmonds and Pitz [1916] have recorded evidence of the presence of a toxic substance in wheat germ and Hart *et al.* [1916] have noted a nervous disorder resembling beriberi in swine, ensuing from a diet rich in wheat products. Rommel and Vedder have also called attention to the marked resemblance between so-called cotton-seed poisoning in swine and human beriberi. That the condition resulting from excessive feeding of cotton-seed is in reality due to poisoning seems to rest on excellent evidence. Taking all these facts into consideration, the most plausible hypothesis by which to account for the harmful effects of large amounts of rice offal on pigeons and swine is that there is present in the outer coats of rice a variable quantity of a more or less distinctly toxic substance. This hypothesis should be tested by further experiment.

These considerations are of especial interest in the suggestions they offer regarding the nature of the "deficiency diseases." How does it happen that what appears to be essentially the same type of nerve lesions is found in beriberi, pellagra and in a mild form in scurvy [McCollum, Simmonds and Pitz, 1916] as well as in animals fed largely on wheat germ, cotton-seed meal or rice offal? Not only are these nerve lesions found in dietary disorders but they have been reliably reported as resulting from oxalic acid [Mauer, 1907], alcoholic and arsenical poisoning [Ross, 1900, 1902].

The writer believes that these facts constitute evidence sufficient to demand consideration that in the "deficiency diseases" there exist toxaemias consequent or incident to dietary disturbances which may play no small rôle in determining the pathology and symptomatology of these diseases. This does not necessarily imply that the foods productive of these diseases are toxic in the ordinary sense. Nor is the opposing argument, that in the case of beriberi the hypothetical toxaemia is corrected by addition to rather than by a subtraction from the diet, necessarily contrary to the view suggested. Hart *et al.* [1916] have shown that effects of the toxic element in wheat which are serious when the diet is otherwise poor disappear almost completely when other dietary defects are corrected.

My thanks are due to Mr G. M. Rommel of the Bureau of Animal Industry, U.S. Dept. of Agriculture, for furnishing part of the necessary rice mill products and also to Lieut.-Col. E. B. Vedder, M.C., U.S.A., for assistance and verification of some of the histological findings.

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CLXXVIII. THE ABSORPTION OF WATER BY GELATIN.

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From the Laboratories of the British Leather Manufacturers' Research Association.

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During the last half-century a large number of papers has been published on the absorption of water by gelatin and the influence on this process of various physical and chemical factors. A review of the literature has recently been given by one of us [Jordan Lloyd, 1926]. Previous work has shown that acids, alkalis, salts, temperature and the relative masses of the gelatin and the water [Jordan Lloyd, 1920; Kuntzel, 1926] all affect the final water content of a gelatin jelly in equilibrium with its surrounding fluid. The correlation between one factor and another is still, however, a fruitful subject for investigation, and this paper provides such a study in the case of a simple system in which all the diffusible ions are univalent. This system is gelatin in the presence of hydrochloric acid, sodium hydroxide, and sodium chloride, over a temperature range from 0° to 24°, a p_H range of 0·5 to 12, and a concentration of sodium chloride from zero to 2·0 mol. per litre. A uniform relation of 100 cc. of fluid to every 0·1 g. of gelatin was used throughout the experiments.

Throughout this paper the term "gel" is used to denote undispersed gelatin, and "jelly" the characteristic 2-phased rigid solution of gelatin in water.

Material.

The gelatin used was prepared from Coignet's "Gold Label" leaf gelatin. Twelve sheets were soaked in 3 litres of 0·2 *M* sodium chloride, adjusted to p_H 3 by the addition of hydrochloric acid; the gelatin was kept in gentle movement, and after 3 or 4 hours the reaction of the fluid was tested and brought back to p_H 3 by the addition of more acid, this process being repeated until equilibrium had been attained. At this stage the whole of the fluid was poured off and replaced by a fresh solution of sodium chloride at p_H 3. This remained on the gelatin overnight, after which a test for calcium always gave a negative result. The gelatin was then dialysed against a stream of distilled water until free from chlorides. The whole preparation lasted a week. During

the last stages of washing the gelatin became white and opaque. The dialysed pieces were dried on coarse net at room temperature. The ash content of the purified material varied from 0·05 to 0·02 % of the dry weight at 100°. This method of purification, which is a modification of one used by Smith [1922], is not so rapid, or so complete, as electrodialysis, but requires no special apparatus. The sodium chloride in the early stages of washing acts as a solvent for any calcium phosphate present and, in addition, inhibits undue swelling of the gelatin in the acid liquors.

Experimental results.

Water absorption under varying conditions was studied by weighing the swollen pieces of gelatin after the attainment of equilibrium with the surrounding fluid, usually after 3 days. The weights of the swollen gelatin are expressed in the final records as a percentage on the dry weight at 100°. The values obtained are not absolute equilibrium figures, since gelatin goes on slowly absorbing water for an indefinite period [Jordan Lloyd, 1920], but they represent the end of the first rapid series of changes due to the influence of the electrolytes on the physical state of the gelatin, the later slow changes being mostly due to hydrolysis. The p_H value of the fluids in equilibrium with the gelatin were all obtained electrometrically. No measurements were made of the possible fixation of sodium chloride by the gelatin.

Three variables influencing water absorption were examined—p_H, temperature (t) and salt content (M). The experimental results are summarised in the curves shown in the figures.

In reviewing the curves shown in the different figures, it should be borne in mind that gelatin is a substance which undergoes a reversible change with change of temperature between two structurally distinct forms known as gelatin A and B [Smith, 1919]. These differ in rotatory power and in solubility, A being nearly insoluble in distilled water, while B has a considerable solubility. In simple gelatin-water systems the whole of the gelatin is present as the A form at temperatures below 15°, and as the B form at temperatures above 35°. The curves shown at 0° and 12° should, assuming for the moment that the presence of the electrolytes does not appreciably affect the proportions of the two forms, indicate systems containing only gelatin A, those at 18° and 24° systems containing mixtures of A and B, although at 18° the proportion of B may be assumed to be very small, while at 24° it will be roughly 50 %. A comparison of the curves in the different figures does actually show very similar behaviour of the systems at 0°, 12° and 18° and a marked contrast at 24°.

The influence of p_H (hydrochloric acid or sodium hydroxide) on the swelling of gelatin at 18° (gelatin A) is shown in Fig. 1. In the absence of inorganic salts the swelling curve has a minimum at p_H 5·0, which may therefore be taken as the isoelectric point, and has maxima in the acid range at p_H 2·6 and in the alkaline range at p_H 9·8. On the alkaline side of the isoelectric

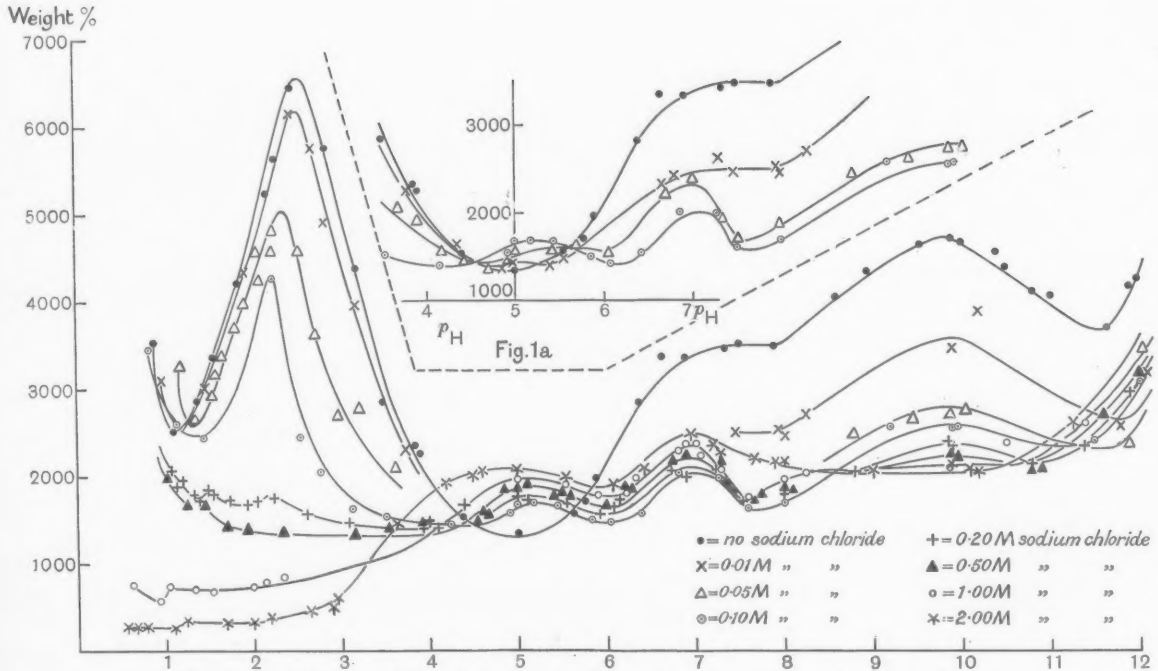

Fig. 1. p_H varying, t constant at 18°, M constant over a series of concentrations from zero to 2·0 M.

point, but close to absolute neutrality (between p_H 7·5 and 7·9), there is an additional broadly defined maximum in the curve of alkaline swelling. At $p_H < 1{\cdot}1$ or $> 11{\cdot}6$ the gelatin dissolves in the external fluid.

It will be convenient to consider separately the behaviour of gelatin in the four zones of hydrogen ion concentration centring respectively on p_H 2·6, 9·8, 5·0 and 7·0. These will be designated as the acid, alkaline, isoelectric and neutral zones.

In acid systems the addition of sodium chloride suppresses the swelling due to the acid, and with increasing concentration ($> 0{\cdot}5$ *M*) actually leads to a coagulation of the gelatin (Figs. 1, 7).

The acid swelling zone (p_H 1·5 *to* 3·5).

The swelling of gelatin gels in acid solutions was first shown by Procter [1914] to be an example of Donnan's well-known phenomenon of membrane equilibrium. The combination of the weak base gelatin with part of the acid present to form soluble, ionisable and hydrolysable salts leads to the production of a non-diffusible cation and one or more diffusible anions and hence to an unequal distribution of the diffusible ions across the gel surface with an excess of diffusible ions inside the gel, the presence of which gives rise to an internal osmotic pressure leading to the absorption of water by the gel. Loeb [1922] has also shown that, between certain limits, the repressive action of metallic salts on the osmotic pressures of gelatin solutions with acid reactions is due to their influence on the membrane equilibrium. Loeb equates the internal osmotic pressure of a gelatin-hydrochloric acid-sodium chloride system to $2y + z - 2\sqrt{y\,(y + z)}$, where z is the concentration of the chlorine ion due to the ionisation of the gelatin chloride, and y the sum of the chlorine ions due to the hydrochloric acid and the sodium chloride. Loeb considers that as y increases while z remains constant, in the limit when y is very large compared to z, the expression $(2y + z)/2\sqrt{y\,(y + z)}$ must equal 1, hence $2y + z = 2\sqrt{y\,(y + z)}$ and the internal osmotic pressure falls to zero. Now when y is large and z is small, the expression $(2y + z)/2\sqrt{y\,(y + z)}$ does not differ greatly from the expression $(y + z)/\sqrt{y\,(y + z)}$. Now $y + z$ is the concentration of the chlorine ions in either a gelatin solution or jelly in equilibrium with an external solution in which the concentration of chlorine ions is $y\,(y + z)$, and Loeb has shown that in the case of a gelatin sol separated from its external fluid by a membrane, a direct titration of the chlorine ions in both the inner and the outer solutions [Loeb, 1922, pp. 183–4] shows that at 25° the value of $(y + z)/\sqrt{y\,(y + z)}$ becomes 1·06, *i.e.* approximates to 1, as the salt concentration reaches 0·062 *M* (*M*/16). Moreover, he shows that if osmotic pressure and the value of the expression $(y + z)/\sqrt{y\,(y + z)}$ be plotted against the logarithm of the salt content, the two curves are parallel (p. 183), both being S curves which ultimately run parallel to the axis of salt concen-

tration. If the effect of the metallic salts on the volume of the jelly is limited, therefore, to an effect on the membrane equilibrium, and if the contribution of the colloidal ions to the internal osmotic pressure is taken as negligible, then it might be anticipated that increasing the concentration of salt up to 0·06 *M* would reduce the jelly to the same volume as in distilled water, after which further increase in salt concentration would have little or no effect. Such is, however, by no means the case. A reference to Figs. 1 and 7 shows that in the acid zone increasing concentration of sodium chloride has an increasing repression on gel volume up to a concentration of 2 *M* and possibly beyond. At 0·5 *M* sodium chloride the swelling in acid solutions is very close to that in distilled water, but at 1·0 and 2·0 *M* the water absorption is far below that in distilled water. The experimental curves showing swelling with varying salt content in acid solutions (Fig. 7) are not of similar form to Loeb's theoretical curve. On the contrary, their general form suggests that they represent a compound function, the sum of two simple functions of *M*, the salt concentration. When $\log M < -0{\cdot}5$ the swelling curve resembles in form the curve of Loeb's equation, but when $\log M > -0{\cdot}5$ the swelling is a negative linear function of $\log M$.

It follows, then, that sodium chloride in acid solutions represses swelling by some additional effect as well as by its influence on the membrane equilibrium. This second effect only becomes patent in comparatively strong solutions (0·5 *M* or greater).

The swelling in the acid zone is strongly influenced by temperature, the volume of the jelly in salt-free systems being apparently an exponential function of t until on the verge of solution (Fig. 2). An acid gel at p_H 2 passes completely into solution at about 20°. This is not due to the appearance of gelatin B in the system, since there is no sudden turning of the volume-temperature curve at about 15–18° as there is in the case of the isoelectric and neutral systems. The influence of temperature on the water absorption of acid gelatin is mainly due to its influence on the ionisation of the weak base gelatin and on the hydrolysis of its salts, with the consequent influence on the membrane equilibrium. A comparison of the acid-binding power of gelatin, as given in the figures of Jordan Lloyd and Mayes [1922] at 20° and of Atkin and Douglas [1924] at 25°, shows that between p_H 5 and 2·5 more acid is fixed at the higher temperature. The temperature effect is greater between p_H 5 and 2·5, at which point saturation of the amino-groups of the gelatin is complete, than between p_H 2·5 and 1. The rotatory power of gelatin salts differs from that of free gelatin [Jordan Lloyd, 1922], but the evidence does not suggest that salt formation is accompanied by a transformation of form A of gelatin into form B. The electrically charged gelatin A as well as gelatin B can form a colloidal solution.

The alkaline swelling zone (p_H 8 *to* 11·5).

It is not as easy to make accurate and reproducible experiments on the properties of gelatin in alkaline solutions as it is in acid solutions. All experiments and estimations of p_H, etc. must be carried out in closed vessels to prevent the absorption of carbon dioxide from the atmosphere. There is, in addition, the difficulty that, even at low temperatures, the hydroxyl ion catalyses the hydrolysis of gelatin very much more than the hydrogen ion. Perhaps for this reason the zone of alkaline swelling has not been studied so much as that of acid swelling. On referring to Fig. 1 it can be seen that the curve of alkaline swelling in pure sodium hydroxide solution is not symmetrical with the acid-swelling curve. As the p_H value moves from 5·0 to 7·0 the volume of the gel rises to a steady level; then, with further increase from 7·9 to 9·8, to a true maximum. With further increase in alkalinity the gel volume decreases up to p_H 11·5, at which value at 18° solution of the gel sets in. Loeb [1922, p. 81] shows a curve of similar form at 25°. The p_H-swelling curve may be compared with the curve of combination with base at varying p_H. Atkin and Douglas [1924], working at 25°, show that the combination of base with protein takes place in two stages, the first complete at p_H 7–8 and the second at p_H 11–12. Although on the acid side p_H 2·6 marks both maximum combination with acid and maximum swelling, on the alkaline side maximum swelling occurs at p_H 9·8, a point which does not correspond with either of the two maxima of combination indicated by Atkin and Douglas. Neither of these maxima, however, is sharply defined, and Reiner [1926] has suggested that the influence of the gelatin anion on the activities of the other ions causes errors in the calculated results. It seems at least highly probable that alkaline swelling with a sharp maximum at 9·8 is due to the formation of salts between the carboxylic groups of the gelatin and the sodium hydroxide, and that the series of sodium gelatinates ionises, with the formation of colloidal anions which lead to an unequal distribution of the diffusible ions between jelly and external fluid, with an excess of diffusible ions in the jelly, leading to an internal osmotic pressure and the absorption of water.

The maximum of alkaline swelling at p_H 9·8 is in all ways the counterpart of the acid peak of swelling at 2·6. The sharpness of the peak, the relative positions of both ascending and descending branches, and the repression of the alkaline swelling by the addition of sodium chloride (Figs. 1 and 7), all suggest that the two systems are similar. The behaviour of sodium gelatinate in the presence of sodium chloride is, however, apparently simpler than that of gelatin hydrochloride. From the curves shown in Figs. 1 and 7 it can be seen that sodium chloride solutions have no coagulating action on alkaline gelatin and apparently the effect of adding this salt in the alkaline zone is limited to its effect on the membrane equilibrium. The repressive action of the salt on swelling seems to reach its full effect in 1·0 *M* solutions. The curve of swelling-log *M* at p_H 10 (Fig. 7) turns and runs parallel to the axis of

log M in accordance with the theoretical expectations implied in Loeb's equation. The volume of the jelly, however, is never brought back to the volume in distilled water, a fact which suggests that the osmotic pressure of the gelatin ions is not negligible.

The effect of temperature on swelling in the alkaline zone is similar to that in the acid zone, and therefore mainly due to the influence of temperature on the ionisation and hydrolysis constants of the gelatin. Fig. 2 shows the effect of temperature on swelling at p_H 10·5. Swelling increases very rapidly with temperature and is, as with acid jellies, an exponential function of t until

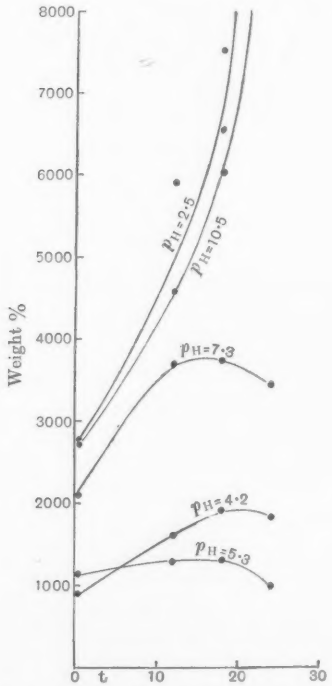

Fig. 2. t varying, M constant at zero, p_H constant at 2·5, 4·2, 5·3, 7·3 and 10·5.

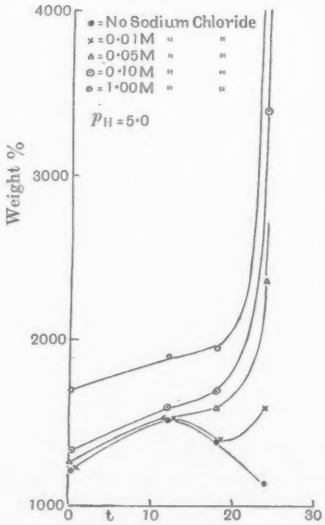

Fig. 3. t varying, p_H constant at 5·0, M constant at five values varying from zero to 1·0 M.

the verge of solution. There is no sign of an abrupt change in the form of the curve between 15° and 18° and the jelly passes into solution at about 20°. The rotatory power of the salts of gelatin with bases differs from that of the free gelatin [Jordan Lloyd, 1922], but the evidence does not suggest that the formation of sodium gelatinates is accompanied by a transformation of the A form of gelatin into the B form. The negatively charged particles of gelatin A form a colloidal solution.

The isoelectric zone (p_H 4·6 *to* 6·0).

The position of minimum swelling in a system free from sodium chloride lies at p_H 4·9 to 5·0, which may be taken as the isoelectric point of the pure gelatin. The presence of sodium chloride in isoelectric or nearly isoelectric solutions causes absorption of water by gelatin A, the amount absorbed at constant temperature being (except in very dilute solutions) proportional to the logarithm of the concentration (Fig. 5, curves at 0°, 12° and 18°). The effect of sodium chloride on water absorption at 24°, when gelatin B may also be assumed to be present, is not directly proportional to log M, but to $e^{\log M}$ (Fig. 5). The p_H of the solution has a marked effect on the influence of the chloride, swelling induced by the action of the salt being greatest at the isoelectric point where the positive and negative gelatin ions are either absent or at a minimum, and increasingly less with increasing concentration of either hydrogen or hydroxyl ions, *i.e.* with increasing concentration of the gelatin ions (Fig. 1). The p_H range over which the swelling effects of sodium chloride are shown is very narrow in dilute solutions, but becomes increasingly wider with increasing salt concentration—at 0·01 M sodium chloride it extends from p_H 4·4 to 5·6 and at 2 M from p_H 3·5 to 6·0 (Fig. 1). The salt influence is evidently limited to the electrically neutral gelatin, and the swelling of gelatin under these conditions is due to an entirely different mechanism from swelling under the influence of the hydrogen or hydroxyl ion.

The effect of temperature on swelling in the isoelectric zone differs strongly from that in the acid and alkaline zones. Below 15° there is a gradual increase of volume with temperature in the salt-free system and at all concentrations of sodium chloride up to 2 M. The influence of the temperature is therefore directly due to action on particles of gelatin A, present in the jelly in the gel form, probably leading to a weakening of the cohesive forces of the rigid structure of the jelly above 15°. The effect of temperature in the absence of salt or at low dilutions (0·01 M) differs from its effect at higher concentrations in an important and striking manner; in the two former cases there is, with further rise of temperature, a decrease in swelling, in the latter a rapid increase (Figs. 2 and 3). This leads to the impression that gelatin B is, in the absence of salt, less hydrated than gelatin A, but is more readily dispersed by sodium chloride. This has already been observed in the smooth but steep rise of the curve showing the influence of salt concentration on swelling at 24° (Fig. 5).

Sodium chloride increases the hydration of electrically neutral gelatin A, but does not disperse it to form a sol. It has a very marked dispersing action on gelatin B.

The zone of neutrality (p_H 6 *to* 8).

Most proteins are isoelectric in solutions which are slightly acid, hence solutions which are neutral, assuming for the moment that neutrality may be defined with sufficient accuracy as any p_H value between 6 and 8, must contain small quantities of base, both free and combined with the protein.

In a system of gelatin jelly and sodium hydroxide free from diffusible salts, an increase of p_H from 5 towards the more alkaline regions leads at first to a fixation of the base by the protein and to a swelling which increases with increasing p_H. At 18° the swelling reaches a steady level between p_H 7·4 and 7·9. At 25°, and at about the same reaction, the combination of protein and base also reaches a steady level. The swelling can therefore be considered as due to the formation of partially ionisable salts. Evidence of the extent of the ionisation is, however, somewhat conflicting. Reiner has pointed out [1926] that immediately on the alkaline side of the isoelectric point there is a zone where there is no migration of protein in an electric field [see also Svedberg, 1924; Cohn, 1925]; hence, therefore, a zone in which there is no ionisation of the protein salt. Experiments here recorded on the influence of salts in the neutral zone (Fig. 1 *a*) suggest that part of the sodium gelatinates is present in the ionised form, since the first effect of adding sodium chloride in the neutral zone is exactly the same as in the acid and alkaline zones, *i.e.* there is a repression of swelling which can be accounted for in the usual manner as due to a readjustment of a membrane equilibrium. In 0·01 *M* solutions of sodium chloride this is the only effect produced by the salt. At 0·05 and 0·10 *M*, although there is increased repression with the increased salt concentration, the curve of swelling has taken on a different form, strongly suggesting that, although the salt is repressing swelling by one chemical mechanism, it is simultaneously promoting it at about p_H 7 by another. At concentrations from 0·10 to 2·0 *M* the effect of the salt is exactly the same as in the isoelectric zone, *i.e.* at 0°, 12° and 18° the swelling of the jelly increases in proportion to the logarithm of the concentration of the salt (Figs. 1, 6 and 7).

The volumes attained at 18° under the influence of salt are rather greater at p_H 7 than at p_H 5, and, although precise figures are not available, it has been ascertained that the melting-point of the jelly is lower at p_H 7. It is an interesting point that, although jellies can swell to about 7000 % of their dry weight under the action of acid or alkali without going into solution, the presence of sodium chloride leads to the conversion of gel into sol if the swelling approaches 3000 %. In the former case only the gelatin ions pass into the sol state, in the latter the electrically neutral gelatin of the gel framework is dispersed.

The influence of temperature on swelling in the neutral zone (Fig. 2, curve at p_H 7·3) shows an interesting compromise between its influence at p_H 5·3 and at p_H 10·5. In the absence of salt and at the lower temperatures, swelling increases nearly as steeply with temperature as in the alkaline zone, at the higher temperature ($t = 18°$) further rise of temperature causes a repression of swelling as in the isoelectric zone. The influence of temperature in the salt-containing systems (Fig. 4) shows again the mixture of two effects. At the lower salt concentrations swelling increases with temperature up to about 20° at 0·01 *M* concentration and to 22° at 0·05, after which point further rise of temperature leads to loss of volume. With increasing salt concentration the

optimum temperature of swelling rises, but after 0·1 M has been reached the curves take on a form resembling those obtained at p_H 5 (Fig. 3).

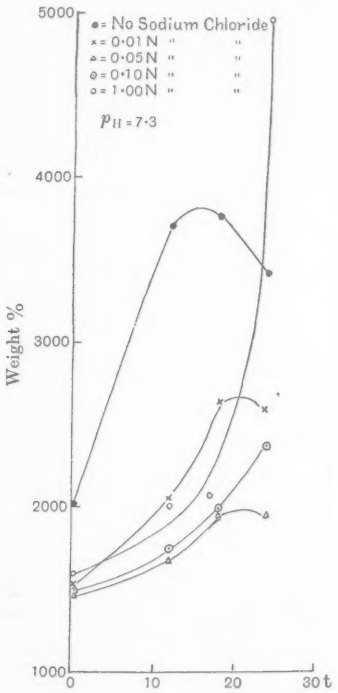

Fig. 4. t varying, p_H constant at 7·3, M constant at five values varying from zero to 1·0 M.

Discussion.

The water content of a gelatin jelly is determined by the balance of a number of factors. The jelly consists of a solid framework of gelatin in the gel form and an interstitial fluid containing one of the several possible sol forms. Unless both sol and gel forms are present the jelly is unstable [Jordan Lloyd, 1920]. Only gelatin A can assume the gel form, but it seems likely that not only the free ampholyte A but also the unionised salts A.HCl and NaA and possibly the hermaphrodite ions $^+A^-$ postulated by Pauli can all take a place in the framework of the jelly. The characteristic of the gel forms of gelatin seems to be the absence of an electric potential. The sol forms of gelatin may be either electrically charged or electrically neutral; in the former case the sol may be formed by free gelatin ions, A^+ and A^-, in the latter by a dispersion of gelatin A through the action of salts, or by gelatin B. The water

content of the jelly is fixed by a balance between osmotic forces in the sol and the elastic force in the gel framework, the latter also containing water present as a solid solution. Reactions which increase the osmotic forces in the interstitial fluid or those which weaken the elastic forces of the framework lead to swelling; conversely, those which decrease the internal osmotic pressure or increase the elasticity lead to shrinking.

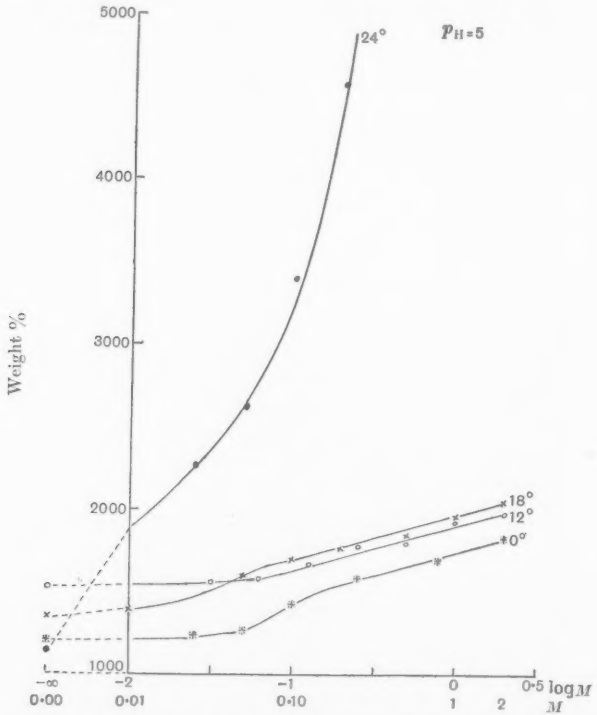

Fig. 5. M varying, p_H constant at 5·0, t constant at 0°, 12°, 18°, 24°.

The formation of ionisable salts between gelatin and hydrochloric acid or between gelatin and sodium hydroxide leads to the appearance in the jelly of colloidal ions with a consequent unequal distribution of diffusible ions across the membrane. The internal osmotic pressure which controls the volume of the jelly is due mainly to the excess in the jelly of the diffusible ions of the gelatin salt, though it can be seen from the results obtained in the alkaline zone that the colloidal ions must contribute some part to the whole. The first effect of the addition of sodium chloride to a system where gelatin hydrochlorides or sodium gelatinates are present in the jelly, is a suppression of swelling which can be accounted for by the readjustment of the membrane

equilibrium. This appears to be the only effect of the salts in the alkaline zone. In the acid zone the sodium chloride, after reaching a concentration of 0·5 *M*, dehydrates and coagulates the gelatin. In saturated sodium chloride solutions the precipitation in the acid zone is complete [Moeller, 1921]. It is tentatively suggested that the excess of sodium chloride dehydrates A^+, the positive gelatin ion, through the greater attraction of its own ion for the dipoles of water. Dokan [1924] has shown that with colloidal carbohydrates strong salt solutions repress swelling apparently by withdrawing the water

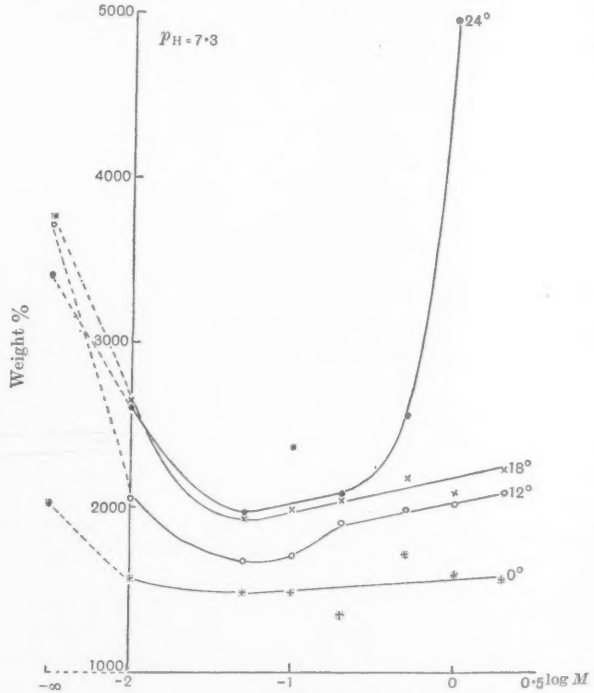

Fig. 6. *M* varying, p_H constant at 7·3, *t* constant at 0°, 12°, 18°, 24°.

from the colloid for the hydration of their own ions. The gelatin cation, which other evidence suggests is heavily hydrated, appears to part fairly easily with its water and is therefore flocculated in concentrated salt solutions. It is especially to be noticed that at a concentration of salt in which neutral or isoelectric gelatin may be assumed to be adsorbing both the positive and negative salt ions with their associated water, thus becoming hydrated, the gelatin cation is giving up its water to these same ions free in the solution. Presumably the gelatin cation does not adsorb the ions of the salt so freely as the uncharged gelatin.

The formation of gelatin salts in a jelly causes swelling by increasing the osmotic forces in the interstitial fluid and simultaneously weakens the framework and the elastic recoil. The effect of change of temperature under these conditions is that swelling increases with rise of temperature. This effect seems mainly due to the influence of the temperature on the ionisation and hydrolysis constants of the gelatin. With rising temperature the hydrolysis of the gelatin salts (sol forms) and regeneration of uncombined gelatin (gel form) will be diminished. Hence, rising temperature will weaken the elastic forces of the framework and at the same time it will increase osmotic pressures through increasing the kinetic energy of both colloidal and diffusible ions in the jelly.

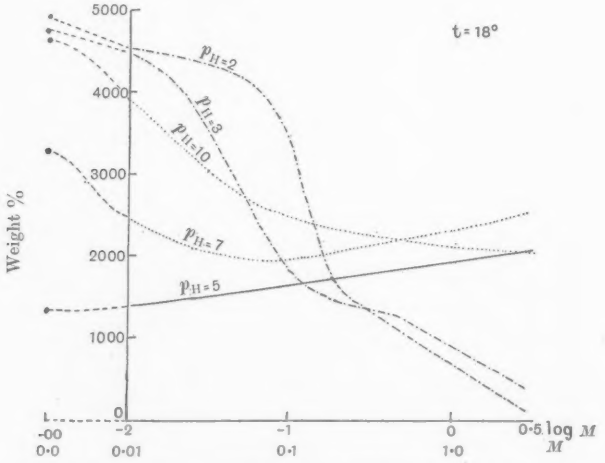

Fig. 7. M varying, t constant at 18°, p_H constant at 2, 3, 5, 7 and 10.

At the isoelectric point the jelly, in equilibrium with water, can be made to swell by a different mechanism from the formation of gelatin salts. At p_H 5 a gelatin jelly free from mineral salts is unstable and the framework collapses under its own forces [Jordan Lloyd, 1920]. With the addition of sodium chloride the conditions for stabilising the jelly appear. The adsorption of both positive and negative ions of the salt by the gelatin, with the resulting hydration of the latter, weakens the elastic forces of the framework. Some of the gelatin will go into the form of an uncharged sol exerting a slight osmotic pressure. Eventually the jelly will be completely dispersed by the salt solution.

Dokan [1924], who has investigated a similar effect of salts on the colloidal carbohydrates, ascribes the action of the salt to the adsorption on to the surface of the electrically neutral colloidal particles of both the positive and negative ions of the salts, each ion carrying into the system its surrounding

shell of water. On this theory the swelling curve should resemble the adsorption curve and be a logarithmic function of the salt concentration, a relation which has been shown to be true for systems consisting mainly of gelatin A.

Temperature has an interesting effect here. A rise of temperature from 0° to 15°–18° slightly weakens the elastic forces of the jelly and the volume increases slightly. At 15°–18° gelatin B, the sol form, begins to appear in the system. Sodium chloride has a strongly dispersing action on gelatin B and salt solutions rapidly dissolve jellies with rise of temperature between 18° and 24°. When no sodium chloride is present, however, rise of temperature over the region 18° to 24° leads to loss of volume by the jelly. Gelatin B, the sol form of uncombined gelatin, is ordinarily more soluble in water than gelatin A, but the latter appears to have a greater capacity of dissolving water in itself to form a solid solution. Further evidence of the behaviour of gelatin in salt-free systems at higher temperatures is needed here.

From the biological point of view the most interesting region studied is the neutral zone, since within this lie the conditions of p_H and salt content generally found in the living cell. The theory of the formation of ionisable sodium gelatinates with a consequent internal osmotic pressure and of the influence of sodium chloride on these, explains the volume relations up to a salt concentration of 0·01 *M*. As the concentration of sodium chloride reaches the order of value found in the living cell (approximately 0·1 *M*) the system is found to have undergone a considerable change. The volume now attained by the jelly, both with change of salt concentration and with temperature, is controlled by the same mechanism as at the isoelectric point, or, in other words, as the salt concentration passes a critical value the gelatin becomes electrically neutral and the jelly volume is now due to a balance of osmotic forces derived from an uncharged sol and the elastic forces of a framework weakened by the adsorption of the hydrated ions of the sodium chloride. The effect of sodium chloride at p_H 7 is greater than at p_H 5. Pauli [1926] considers that at the isoelectric point solutions of proteins may contain the protein in four different forms: the charged ions A^+ and A^-, the uncharged pseudo-ampholyte A and the hermaphrodite ion of the internal salt form $^+A^-$. The first additions of acid are said to increase the concentrations of $^+A^-$. It must remain for the present an interesting speculation whether the first additions of alkali have a similar but possibly even greater effect. Other salts are now under investigation in order to provide further evidence for or against the hypotheses advanced in the paper.

SUMMARY.

1. Sodium chloride, in the presence of hydrochloric acid and up to a concentration of 0·01 *M*, suppresses swelling of gelatin due to the acid; at greater concentrations it causes a coagulation of the acid gelatin. The maximum of acid swelling is at p_H 2·6.

2. Sodium chloride in the presence of sodium hydroxide suppresses the swelling of gelatin due to the alkali. Even at high concentrations (2 *M*) the salt has no further action. Maximum alkaline swelling is at p_H 9·8.

3. The swelling of gelatin due to acid or alkali increases as an exponential function of the temperature.

4. The point of minimum swelling of ash-free gelatin (isoelectric point) is at p_H 5·0. At this point sodium chloride promotes water absorption, swelling being proportional to the logarithm of the concentration of the salt.

5. At p_H 5, in the absence of salts, swelling increases slowly with rising temperature up to 15° or 18°, after which, with further rise, there is a decrease of swelling.

6. At p_H 5 the presence of sodium chloride increases swelling slowly with temperature up to about 18°, after which there is very rapid increase ending at about 20° in complete solution of the gelatin. The greater the concentration of sodium chloride present, the lower the temperature at which solution occurs.

7. At p_H 7, in the absence of salts, gelatin shows some swelling due to the alkali present. The addition of sodium chloride up to 0·01 *M* causes a suppression of swelling; at greater concentrations the salt, as at p_H 5·0, induces a swelling which is in logarithmic ratio to its concentration.

8. At p_H 7, in salt solutions stronger than 0·01 *M*, the effect of temperature on swelling resembles its effect at p_H 5. When the concentration of sodium chloride is less than 0·01 *M*, or in the absence of salts, the effect of temperature on swelling at p_H 7 is intermediate between its effects in the alkaline and in the isoelectric zones.

9. When gelatin is in solution as electrically charged particles the effect of adding sodium chloride is mainly electrostatic; when present in electrically neutral forms, the salt acts by adsorption and hydration.

10. The theoretical aspects of swelling are discussed.

In conclusion we should like to thank the Council of the British Leather Manufacturers' Research Association for permission to publish this work and the Director (Dr R. H. Pickard, F.R.S.) for the interest and criticism which he has given us during its progress.

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CLXXIX. NOTE ON THE ATTEMPTED ACTIVATION OF TYROSINE BY ULTRA-VIOLET IRRADIATION.

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(*Received October 31st, 1927.*)

In the course of a recent paper on the ultra-violet irradiation of rations Waltner [1927] has claimed that pure tyrosine may be rendered antirachitic by such treatment. Although this statement finds no support in the theory of specific sterol activation now generally accepted, the importance of tyrosine in melanin formation on the one hand, and the relation of protective pigmentation to irradiation on the other, led us to put the matter to independent test. As a result we are unable to confirm the view that tyrosine may be rendered antirachitic.

Experimental.

A sample of tyrosine, to ensure freedom from impurities of a fatty nature, was extracted with ether for 48 hours in a Soxhlet apparatus. One portion was then exposed in a thin layer before a mercury arc lamp for a period of 20 minutes at a distance of 50 cm., the remainder was retained for use in the negative control diet. For use in the positive control diet a sample of ergosterol supplied by the British Drug Houses was exposed at the same time as the tyrosine to the radiations of the same lamp.

In order to eliminate the possibility of fortuitous healing through fall of food intake, etc., it was decided that the curative method, employed by Waltner, should not be adopted, but that the materials should be tested by their efficiency in preventing rickets when introduced into the diet from the outset. Twelve young albino rats were divided into three groups. Two rats (Group I) received the McCollum diet 3143 with the daily supplement of 0·1 mg. of irradiated ergosterol suspended in liquid paraffin; five rats (Group II) received diet 3143 with the admixture of 0·5 % of irradiated tyrosine; the remaining five received the same diet containing a similar amount of tyrosine which had not been irradiated. After 45 days the rats were killed and their bones examined, with the results shown in the table.

It will be seen that whereas almost normal bones were observed in the case of the two rats which had received irradiated ergosterol, no distinction in the severity of rickets could be drawn between the remaining two groups,

symptoms in the case of rats which had received irradiated tyrosine being as gross as in the case of rats which had received the material without irradiation. Therefore, while such results do not rule out the possibility that irradiated tyrosine may have some slight healing effect on rickets in its early stages, it is obvious that its continued administration cannot prevent or moderate the progress of rickets in the case of the rat.

Table I.

	Weight (g.)	State of ribs	Diagnosis
Group I (irradiated ergosterol).			
♂	42–92	Normal for 3143 diet	Normal
♀	39–74	Normal for 3143 diet	Normal
Group II (irradiated tyrosine).			
♀	47–92	Costochondral junctions blurred. Three ribs fractured. Marked angulation	Severe rickets
♀	49–72	Junctions much enlarged with cupping, lines blurred. Four ribs fractured. Some angulation	Severe rickets
♂	39–90	Junctions much enlarged, lines fairly distinct. Two ribs fractured. Marked angulation	Severe rickets
♂	45–82	Junctions enlarged with lines slightly blurred. Four ribs fractured. Slight angulation	Severe rickets
♂	49–76	Junctions almost normal, but two ribs fractured. No angulation	Slight rickets
Group III (non-irradiated tyrosine).			
♀	40–71	Junctions enlarged and cupped, lines blurred. Four ribs fractured with partial collapse of thorax on left side	Very severe rickets
♂	44–61	Junction lines fairly distinct. Eleven ribs fractured. Severe angulation	Very severe rickets
♂	46–109	Junction lines fairly distinct. One rib fractured. Angulation on left side	Moderate rickets
♂	49–116	Junction lines rather blurred. Very slight angulation	Slight rickets
♀	52–102	Junctions slightly enlarged and lines rather blurred. No angulation	Very slight rickets

SUMMARY.

Irradiated tyrosine admixed at the rate of 0·5 % in the McCollum diet 3143 is ineffective in moderating the course of rickets in rats.

Our thanks are due to Sir F. G. Hopkins for his support and criticism and to Prof. Drummond for the use of a mercury arc lamp.

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CLXXX. THE ACTION OF TYROSINASE ON PHENOLS.

WITH SOME OBSERVATIONS ON THE CLASSIFICATION OF OXIDASES.

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(*Received October 31st, 1927.*)

It has been known for many years that tyrosinase will bring about the oxidation of certain phenols, and the reaction with *p*-cresol has been used by Chodat [1912] as an effective means of identifying the enzyme. In the presence of amino-acids the reaction with *p*-cresol produces a series of brilliant colour changes, and in addition deamination of the amino-acid takes place. It was shown by Happold and Raper [1925], and almost at the same time by Robinson and McCance [1925], that the deamination was not due, as Chodat thought, to a direct action of tyrosinase on the amino-acid, but to the intervention of the phenol in the reaction. Happold and Raper [1925] brought forward evidence that *ortho*-quinones were first produced from those phenols which were attacked by tyrosinase, and that the deamination produced in the system, tyrosinase-phenol-amino-acid, was due to action of the *o*-quinone thus produced on the amino-acid.

It was shown at that time that *o*-benzoquinone would bring about the deamination of alanine and glycine under the same conditions as to reaction of the medium and temperature, which prevailed in the enzyme action. This evidence has been extended in the present paper by showing that *o*-homoquinone behaves in a like manner towards glycine. Additional indirect evidence of the formation of *o*-quinones from phenols by the action of tyrosinase has been put forward by Raper [1927] in explaining the action of this enzyme on tyrosine. In the series of changes which take place in this reaction, and which result finally in the production of melanin, it seems very probable that the 3.4-quinone of phenylalanine is one of the intermediate products. It has been shown by Onslow and Robinson [1925] that the system *p*-cresol-tyrosinase, and by Szent-Györgyi [1925] that *o*-quinone, turns tincture of guaiacum blue, and since the product of the action of tyrosinase on catechol

and certain other phenols will turn guaiacum blue it has been suggested by the latter that *o*-quinones are produced by the action of the enzyme on these phenols.

Since all this evidence is indirect it seemed desirable to establish it more securely, if possible, by the isolation of compounds of *o*-quinone from the reaction system. *o*-Benzoquinone is a very unstable substance and can be prepared only in dry ether, and *o*-homoquinone is not much more stable. It seemed likely therefore that these substances, if produced by the action of tyrosinase on phenol or *p*-cresol, would have only a transient existence, and that to isolate them some substance must be used to withdraw them from the sphere of reaction. Aniline, upon which tyrosinase has no action, was found to be suitable since it forms with quinones insoluble anilinoquinones. Benzenesulphinic acid, which has been shown by Hinsberg and Himmelschein [1896] to combine with *o*-benzoquinone, was also tried instead of aniline with the hope of being able to isolate such a compound from the products of the action of tyrosinase on phenol and on catechol. It was however successful only with catechol.

The anilinoquinones produced, as described above, by the action of tyrosinase on phenol, catechol, *p*-cresol, *m*-cresol, and homocatechol in presence of aniline, have been isolated, and identified by synthesis. It has also been shown by the same method that peroxidase and hydrogen peroxide acting on catechol and on homocatechol produce *o*-benzoquinone and *o*-homoquinone respectively. It seems, therefore, to be clear that the first stages in the action of tyrosinase on those monohydric phenols on which it acts are the introduction of a second hydroxyl group in the *o*-position to the first, thus producing a catechol derivative, and then oxidation of this to the corresponding *o*-quinone. Peroxidase and hydrogen peroxide also bring about the latter reaction.

Not all phenolic substances which are attacked by tyrosinase give rise to anilinoquinones when the enzyme acts on them in the presence of aniline, but *m*- and *p*-methoxyhydroxybenzene do so. Tyrosine and pyrogallol are examples of substances which, though oxidised by the enzyme, do not yield anilino-compounds under these conditions. There is good evidence, however, that in both these instances *o*-quinones are produced, but, owing to further reactions which these undergo, *i.e.* the formation of indole derivatives from tyrosine [Raper, 1927], and of purpurogallin from pyrogallol [Willstätter and Heiss, 1923], combination with aniline does not take place.

By means of the microrespirometer, some indication has been obtained of the quantitative relations which hold between the amounts of oxygen taken up and of phenols oxidised. The results show that in those instances in which it has been proved that *o*-quinones are formed, the oxygen taken up is greater than the amount required for the formation of the *o*-quinone. The enzyme preparation used in these experiments contained catalase, so that the excess oxygen uptake over and above that which is required for the

formation of the *o*-quinone, cannot be accounted for simply by the formation of hydrogen peroxide as represented, for example, in the reaction

$$C_6H_4(OH)_2 + O_2 = C_6H_4O_2 + H_2O_2,$$

in which oxygen is acting as a hydrogen acceptor. It would appear that the *o*-quinones take part in some further reaction or reactions which involve oxidation. The nature of these has not yet been discovered.

The results described in this paper together with those obtained by other workers in this field during the past few years suggest that the hypothesis as to the nature of the "direct oxidases" which was put forward by Bach and Chodat [1903] requires revision. The "direct oxidases" are usually detected by the fact that they will turn guaiacum tincture blue without the addition of hydrogen peroxide or other peroxides. This distinguishes them from peroxidases or "indirect oxidases" which only give this reaction when peroxides are added. Bach and Chodat believed that peroxidases were an essential component of the "direct oxidases," and that the reason why the latter turned guaiacum blue directly was that a second component was present which in presence of air produced a peroxide. Thinking that this second component was enzyme-like in its action they named it oxygenase. But we now know that peroxidase is not an essential component of the "direct oxidases." It has been shown by Szent-Györgyi [1925] that *o*-quinone will turn guaiacum blue, and in the present paper proof is presented that *o*-quinones are produced by the action of tyrosinase on certain phenols, some of which, *e.g.* catechol and some of its derivatives, are known to be present in plants. Furthermore, thermostable catalysts known as polyphenolases have been prepared from plant products by Euler and Bolin [1908] and Wieland and Fischer [1926], which oxidise dihydric phenols to quinones with the formation of hydrogen peroxide. In the presence of catechol or some catechol derivative and even in the absence of peroxidase, these catalysts would cause the direct blueing of guaiacum because of the production of *o*-quinones. Since these systems give the guaiacum reaction it has therefore ceased to be an accurate guide to the presence of a "direct oxidase" of the kind postulated by Bach and Chodat. Altogether four different oxidation systems have now been shown to give the "direct oxidase" reaction.

(1) Peroxidase + an autoxidisable substance which produces hydrogen peroxide or an organic peroxide in air. Gallagher [1923] has found a system of this type in the potato, the autoxidisable substance being a lipin or a mixture of lipins. It seems likely that many unsaturated substances, *e.g.* terpenes or unsaturated fats or fatty acids would behave likewise. Bertrand's laccase probably comes into this group.

(2) Peroxidase + an oxidoreductase and its substrate. An example of this system is that investigated by Thurlow [1925] in her studies of the xanthine oxidase of milk, which, when acting on xanthine or hypoxanthine in presence of air, gives rise to the production of hydrogen peroxide.

(3) Peroxidase + a polyphenolase with a polyphenol as substrate. Euler and Bolin [1908] have found a system of this type in *Medicago sativa*. Their polyphenolase was thermostable and was apparently a mixture of calcium salts of organic acids. Its action could be imitated by a mixture of calcium glycollate and manganese acetate. If the polyphenolic substrate is a catechol derivative, the peroxidase is not necessary to give the "direct oxidase" reaction, since the *o*-quinone produced by the action of the polyphenolase would blue guaiacum by itself. In this group would come also the thermostable polyphenolase isolated by Wieland and Fischer [1926] from *Lactarius*. This was different in composition from that obtained by Euler and Bolin but exerted the same type of action.

(4) Tyrosinase + one of its substrates (tyrosine, 3 . 4-dihydroxyphenylalanine and pyrogallol excepted). This is the system described in the present paper.

It is clear from the composition of these various systems that the term "direct oxidase" is a misnomer if, by it, we wish to denote only one particular oxidising enzyme or oxidation system, and it would be better to abolish it. The "direct oxidase" reaction is essentially a test for the presence of those oxidases and thermostable oxidation catalysts which will bring about oxidation only in the presence of oxygen or peroxides, and the authors suggest that they be called *aerobic oxidases*. These agents are different from the oxidoreductases, which will act in the absence of oxygen provided a hydrogen acceptor is present, and which may be called *anaerobic oxidases*. The oxidoreductases are believed to act by activating hydrogen, but whether this is also true of the aerobic oxidases is at present uncertain. Wieland and Fischer [1926] suggest that the polyphenolase which they have investigated acts by activating hydrogen and not by activating oxygen in the sense that certain metallic ions are supposed to do. Their suggestion of the mechanism is that the catalyst, polyphenol and molecular oxygen unite to form an unstable compound which then breaks down, giving the quinone and hydrogen peroxide. Whether tyrosinase acts in this way is not yet decided in view of the lack of experimental evidence; but Willstätter and Weber [1926] explain the action of peroxidase by the formation of a compound between the enzyme and hydrogen peroxide in which the oxygen is more active than in hydrogen peroxide itself. Although it is clear therefore that the aerobic oxidases act only in the presence of oxygen or a peroxide, it is by no means decided whether they activate oxygen or hydrogen.

In addition to the hypothesis regarding the constitution of the "direct oxidases" which was put forward by Bach and Chodat, an alternative scheme has been suggested by Onslow [1920]. It is that the oxygenase of Bach and Chodat is really a mixture of an enzyme proper—to which Onslow applies the name oxygenase—and a catechol derivative. This view was based essentially on the separation from plants that give the "direct oxidase" reaction, of an alcohol-insoluble enzyme and an alcohol-soluble substance which gave

the typical green colour with ferric chloride which is characteristic of catechol derivatives. Neither of these alone gave a definite "direct oxidase" reaction, but when combined gave it very markedly. The results described in the present paper, however, show that tyrosinase has all the properties ascribed by Onslow to oxygenase and the system she describes is really that of tyrosinase with a catechol derivative. The polyphenolases of Euler and Bolin and of Wieland and Fischer would also give Onslow's oxygenase reaction. There is no evidence, therefore, that this is a new enzyme, and it seems unnecessary to retain the term oxygenase in the sense used by Onslow.

Nor does this term convey a precise meaning in the sense in which Bach and Chodat first employed it, because, as shown above, it may represent a different component in each one of the four known "direct oxidase" systems.

It is suggested, therefore, that in view of the present state of our knowledge, oxidases should be provisionally divided into an aerobic and an anaerobic group. Those of the former act only in the presence of oxygen or a peroxide, and comprise peroxidase, tyrosinase, and the thermostable catalysts known as polyphenolases. Those of the latter include the known oxidoreductases, and the thermostable catalysts which act in conjunction with glutathione. They will act anaerobically provided a suitable hydrogen acceptor is present.

Experimental.

The enzyme used in this investigation was prepared from mealworms as described by Raper [1926]. In experiments in which enzyme was used, chloroform (unless otherwise stated) was added as antiseptic. The p_H of solutions was adjusted by means of phosphate buffer.

The isolation of anilino-o-quinones.

o-Quinones being highly reactive and unstable substances, it was not expected that it would be possible to isolate them as such from solutions of phenols which had been submitted to the action of tyrosinase. Attempts were therefore made to remove them from the sphere of reaction by introducing aniline into the system and isolating them as anilinoquinones. It was found that by the action of tyrosinase on phenol in presence of aniline a bright red precipitate was obtained, which crystallised from ether as bright red needles having the characteristics of an anilinoquinone. Tests showed that a maximum yield of this deposit was obtained at p_H 6·5–7·0. The experiments were therefore carried out at p_H about 6·7. The action of tyrosinase in presence of aniline was investigated first of all on phenol and on catechol, then on the cresols and on homocatechol.

Controls. A preliminary control showed that aniline in phosphate buffer at p_H 6·7 is unchanged by tyrosinase. Phenol in buffered aniline water remained unchanged. Catechol became slowly pink, and after some time gave a reddish brown precipitate, this being due presumably to autoxidation to

o-benzoquinone which then reacts with the aniline. In presence of hydrogen peroxide the oxidation was more rapid. *p*-Cresol and *m*-cresol in buffered aniline water remained unchanged. Homocatechol underwent fairly rapid oxidation, a red substance and some tar being produced by reaction with aniline. The oxidation was hastened by hydrogen peroxide, but in no case was the product obtained nearly so rapidly or definitely as when enzyme was present.

Buffered solutions of phenol and catechol became darkened by the action of tyrosinase, and catechol was also darkened by the action of peroxidase and hydrogen peroxide. Buffered solutions of *p*-cresol, *m*-cresol, and homocatechol by the action of tyrosinase, and of homocatechol by the action of peroxidase and hydrogen peroxide, became orange-yellow, orange-brown, or reddish brown.

Action of tyrosinase on phenol in presence of aniline.

6 g. phenol and 12 cc. aniline were dissolved in about 1·5 l. of buffer solution, and 120 cc. tyrosinase solution were added. The liquid was placed in two 2 l. flasks, and gently aerated with air drawn through a wash-bottle containing chloroform and water. In a few minutes a red colour developed, the liquid clouded, and later red crystalline needles separated out. After two days the precipitate was filtered off on fluted filters, washed with 1 % HCl to remove excess aniline, then with water, and dried *in vacuo*. In cases where the filtrate was still reddish coloured, a further yield was obtained by acidification with 1 % HCl, or precipitation with $CaCl_2$ and 1 % NH_4OH, or by extraction with ether and evaporation of the ether. The total dried substance was extracted in a Soxhlet apparatus with acetone or light petroleum. The latter was slower in its solvent action. On evaporation of the solution bright red needles separated out. The product was purified by recrystallisation from acetone or light petroleum, or alternatively, by solution in ether and extraction with 0·02 *N* NaOH, the aqueous liquid being acidified with 2 % HCl, and the red precipitate filtered off and crystallised from acetone. Yield, 4·5 g. Properties: bright red needles; M.P. 193°; soluble in organic solvents; soluble in concentrated sulphuric acid, giving an olive green colour, becoming red on dilution by reprecipitation; soluble in alkali and alkali carbonate, giving a purplish red solution, reprecipitated by acid.

Analysis. Found: C, 74·58 %; H, 5·08 %; N, 9·57 %.
Calculated for dianilino-*o*-benzoquinone, $C_{18}H_{14}O_2N_2$:
C, 74·48 %; H, 4·83 %; N, 9·66 %.

Action of tyrosinase on catechol in presence of aniline.

2 g. catechol, 4 cc. aniline, and 40 cc. tyrosinase solution were made up to nearly a litre in buffered solution, and aerated. Most of the precipitate was obtained the first day. The product was treated in the same way as that

obtained from phenol. Yield, 1·75 g. Properties: identical with those of the product obtained from phenol; M.P. 193°. Mixed M.P. with the product obtained from phenol, 193°.

Analysis. Found: N, 9·61 %.
Calculated for $C_{18}H_{14}O_2N_2$: N, 9·66 %.

Action of peroxidase and hydrogen peroxide on catechol in presence of aniline.

As was expected, catechol is oxidised by peroxidase and hydrogen peroxide in presence of aniline with production of the same substance as is obtained by the action of tyrosinase on phenol and on catechol in presence of aniline. The peroxidase used was prepared by slicing horseradish into chloroform water, avoiding long exposure to air, and rejecting the outer portions. Only preparations were used which remained practically unchanged by aniline water in presence of hydrogen peroxide. 2 g. catechol and 4 cc. aniline were made up to nearly a litre, about 300 cc. peroxidase and 70 cc. 1 % hydrogen peroxide were added. A red precipitate appeared very rapidly. The product was treated in the same way as that obtained from phenol. Yield, 1·15 g. Properties: identical with those of the product obtained from phenol; M.P. 193°. Mixed M.P. with the product obtained from phenol, 193°; with that from catechol by tyrosinase, 192·5°.

Analysis. Found: N, 9·63 %.
Calculated for $C_{18}H_{14}O_2N_2$: N, 9·66 %.

Synthesis of dianilino-o-benzoquinone.

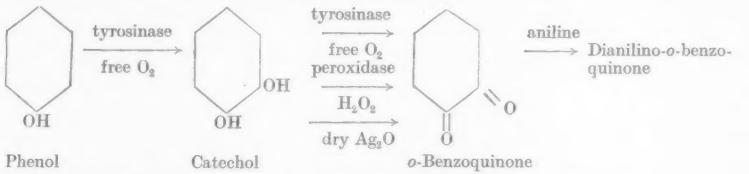
o-Benzoquinone in ethereal solution was synthesised by the method of Willstätter and Müller [1908] using 2·5 g. catechol. The ethereal solution was poured into a solution of 6 cc. aniline in 1700 cc. water, and shaken. A red precipitate separated out, which was filtered off. It was treated in the same way as the product obtained from phenol. Properties: identical with those of the products obtained from phenol and catechol; M.P. 193·5°. Mixed M.P. unchanged.

Analysis. Found: C, 74·47 %; H, 5·05 %; N, 9·67 %.
Calculated for $C_{18}H_{14}O_2N_2$: C, 74·48 %; H, 4·83 %; N, 9·66 %.

This proves the identity of the products obtained by enzyme action with dianilino-*o*-benzoquinone, and indicates therefore that *o*-benzoquinone is produced from phenol and from catechol by the action of tyrosinase, and from catechol by the action of peroxidase and hydrogen peroxide.

Dianilino-*o*-benzoquinone has been synthesised by Kehrmann and Cordone [1913] by oxidising catechol with silver oxide in glacial acetic acid solution containing aniline, the product being poured into water. The properties described are identical with the above.

The reactions taking place in the experiments described above may therefore be represented as follows:



Phenol Catechol *o*-Benzoquinone

Action of tyrosinase on p-*cresol in presence of aniline.*

6 g. *p*-cresol, 15 cc. aniline, and 100 cc. tyrosinase solution were made up to about a litre in buffered solution and aerated. The liquid became reddish, then a brownish red precipitate appeared. The major part of the substance remained too finely divided to be filtered off, so it was coagulated, or else extracted, as described for the filtrate in the case of phenol. The product was washed, dried, and extracted and crystallised by means of acetone. Some tarry substance was produced besides the anilinoquinone. The extraction was therefore fractionated; the tar, being exceedingly soluble in acetone, was removed in the earlier portions of the extract. Yield, about 1 g. of crystals. Properties: brownish red needles; M.P. 202°; soluble in organic solvents, but much less soluble in light petroleum than the product obtained from the lower homologue; soluble in concentrated sulphuric acid, giving a brownish crimson colour, becoming brown then yellow on dilution; insoluble in alkali.

Analysis. Found: C, 79·15 %; H, 5·55 %; N, 10·92 %.

Calculated for dianilinohomoquinoneanil, $C_{25}H_{21}ON_3$:
C, 79·15 %; H, 5·54 %; N, 11·08 %.

Action of tyrosinase on m-*cresol in presence of aniline.*

6 cc. *m*-cresol, 15 cc. aniline, and 100 cc. tyrosinase solution were made up to about a litre in buffered solution, and aerated. The product was treated in the same way as that obtained from *p*-cresol. Yield, less than 1 g. owing to the tarry nature of part of the extract. Properties: identical with those of the product obtained from *p*-cresol; M.P. 201·5°. Mixed M.P. unchanged.

Analysis. Found: C, 78·75 %; H, 5·57 %; N, 11·01 %.

Calculated for $C_{25}H_{21}ON_3$: C, 79·15 %; H, 5·54 %; N, 11·08 %.

Action of tyrosinase on homocatechol in presence of aniline.

The homocatechol used was prepared from creosol by the method of de Vries [1909], the creosol being prepared from vanillin. 1 g. homocatechol, 3 cc. aniline, and 40 cc. tyrosinase solution were made up to 150 cc. in buffered solution and aerated. The action was rapid and the yield remarkably good—about 0·9 g.—very little tar being obtained. Properties identical with those of the product obtained from *p*-cresol; M.P. 202°. Mixed M.P. with synthetic product (*vide infra*), 202°.

Analysis. Found: N, 10·80 %.

Calculated for $C_{25}H_{21}ON_3$: N, 11·08 %.

Action of peroxidase and hydrogen peroxide on homocatechol in presence of aniline.

0·5 g. homocatechol and 1·5 cc. aniline were dissolved in about 100 cc. buffer solution, and 90 cc. peroxidase and 18 cc. 2 % H_2O_2 added. The liquid became red and clouded, but no precipitate appeared. It was extracted with ether, the extract was dried over anhydrous sodium sulphate, filtered, and the filtrate evaporated, crystals being obtained which were recrystallised from acetone. Properties: identical with those of the product obtained from *p*-cresol; M.P. 201°. Mixed M.P. with the product obtained from *m*-cresol, 201°.

Analysis. Found: N, 11·04 %.

Calculated for $C_{25}H_{21}ON_3$; N, 11·08 %.

Synthesis of dianilinohomoquinoneanil.

The following method was found to obviate the production of tar. Homoquinone in ethereal solution was synthesised by the method of Willstätter and Müller [1911], using 6 g. homocatechol. The solution was dropped slowly into a solution of 20 cc. aniline in water, mechanically stirred. The liquid became purplish, then almost black. On standing overnight, it became cloudy and brick red, and deposited a tarry solid. The lumps of solid matter which had separated were washed with a little ether and crystallised from acetone. A further yield was obtained by extracting the liquid with ether. This extract was dried over anhydrous sodium sulphate, filtered, and the filtrate evaporated on the water-bath, then left to stand in the vacuum desiccator, when crystals were deposited which were recrystallised from acetone. Total yield, about 2·5 g. Properties: identical with those of the products obtained from *p*-cresol, *m*-cresol, and homocatechol; M.P. 202·5°. Mixed M.P. unchanged.

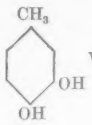
Analysis. Found: C, 79·01 %; H, 5·67 %; N, 11·35 %.

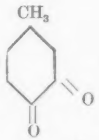
Calculated for $C_{25}H_{21}ON_3$: C, 79·15 %; H, 5·54 %; N, 11·08 %.

The synthesis of dianilinohomoquinoneanil was also carried out by oxidising homocatechol with silver oxide in glacial acetic acid in presence of aniline, the product being poured into water. A method was also used analogous to that of Jackson and Koch [1898] for the preparation of dianilinoquinoneanil; homoquinone with aniline in chloroform was boiled on the water-bath for 10 minutes. The substances obtained by both these methods were identical with that obtained by the action of an ethereal solution of homoquinone on aniline water.

The identity of the products obtained by enzyme action with dianilinohomoquinoneanil is thus proved, and this indicates that homoquinone is produced from *p*-cresol, *m*-cresol, and homocatechol by the action of tyrosinase, and from homocatechol by the action of peroxidase and hydrogen peroxide.

The reactions taking place in the experiments just described may therefore be represented in a precisely similar manner to those with phenol (p. 1377).

m- and *p*-Cresol yield the same homocatechol  which then passes into

homoquinone  and dianilinohomoquinoneanil.

The action of tyrosinase on other phenols.

The action of tyrosinase was tried on a number of other phenolic substances alone and in the presence of aniline. The following observations were made at p_H 6·5 or 7·0 after a period of 1 day, the phenols used being at concentrations of about 0·2 % or 0·1 % or, if only very slightly soluble, in saturated solution.

Substrate used	Substrate in buffer alone	Substrate in buffer with tyrosinase	Substrate in buffer with tyrosinase and aniline water	Substrate in buffer with aniline water
Quinol* (p_H 7·0)	Pink	Dark brown	Yellow	Yellow
" (p_H 6·0)	Faintly pink	"	"	"
Pyrogallol	Pale yellow	Darkened Brownish ppt. giving purpurogallin reaction with ammonia	Darkened Brownish ppt. No anilinoquinone	Pale yellow
m-Methoxyhydroxybenzene	Nil	Reddish brown	Bright red ppt. of anilinoquinone	Very faintly coloured
p-Methoxyhydroxybenzene	Nil	Reddish brown	Bright red ppt. of anilinoquinone	Very faintly coloured
p-Aminophenol	Brown ppt.	Brown ppt. Darkened	Brown ppt. Darkened	Brown ppt.
Tyrosine	Nil	Dark brown	Brown. No anilinoquinone	Nil

* The oxidation of quinol appears to be inhibited unless the solution is dilute and the enzyme very active. A similar phenomenon may possibly occur with guaiacol. The matter has not yet been fully investigated. The yellow colour with quinol and aniline is due to an addition compound.

No reactions were observed with the following: resorcinol, phloroglucinol, guaiacol, *o*-cresol, orcinol, creosol, eugenol, *iso*-eugenol, *o*-, *m*-, and *p*-nitrophenol, and thymol. Vanillin and salicylaldehyde reacted as aldehydes with aniline, the other tests being negative.

Thus the only further substances found to give rise to anilino compounds were *m*- and *p*-methoxyhydroxybenzene, which were attacked apparently in an analogous manner to *m*- and *p*-cresol. In presence of aniline bright red substances were obtained, having the properties of an anilinoquinone: they were soluble in concentrated sulphuric acid, giving a brownish colour, reprecipitated on dilution; soluble in alkali, giving a purplish red solution, reprecipitated on acidification.

Action of tyrosinase on catechol in presence of benzenesulphinic acid.

The addition product of *o*-benzoquinone with benzenesulphinic acid was obtained by Hinsberg and Himmelschein [1896] by the oxidation of catechol in presence of benzenesulphinic acid.

An attempt was made to obtain this compound by the action of tyrosinase on phenol, and also on catechol, in presence of benzenesulphinic acid. The attempt was successful in the case of catechol, but not in that of phenol. *o*-Dihydroxydiphenylsulphone (M.P. 165°) was found to be very feebly soluble in benzene, in which phenol, catechol, and benzenesulphinic acid are all readily soluble. The product of action of tyrosinase on catechol in presence of benzenesulphinic acid was isolated by making use of these facts.

0·2 g. catechol and 0·3 g. benzenesulphinic acid were dissolved in water, and the p_H adjusted with phosphate buffer to 6·0–6·5. The solution was made up to 100 cc., 3 cc. tyrosinase solution were added, and the liquid was allowed to stand for a few days, with frequent shaking. It was then extracted with ether and the ether evaporated. The residue was boiled under reflux with benzene for a minute or two and the white residue filtered off. This was dissolved in boiling water, from which it separated as crystals on cooling. The crystals were dried in a desiccator over concentrated sulphuric acid *in vacuo*, water of crystallisation being thereby removed. Properties: white powder, M.P. 164°; mixed M.P. with *o*-dihydroxydiphenylsulphone, unchanged; aqueous solutions of both gave a blue-green colour with ferric chloride, changed to red by sodium carbonate.

Several attempts, including the use of increased aeration, were made to obtain the same substance by the action of tyrosinase on phenol in presence of benzenesulphinic acid; but in every case the residue on evaporation of the ether was readily entirely soluble in benzene in the cold. Thus there is no evidence that tyrosinase will oxidise phenol in presence of benzenesulphinic acid. It is possible that a higher oxygen potential is required for the oxidation by tyrosinase of phenol than of a catechol, and that the reducing action of benzenesulphinic acid prevents this potential from being reached. But this can be determined only by further experiment.

Deamination of glycine by homoquinone.

The method used was essentially that described by Happold and Raper [1925], a funnel being used to run in the solutions, and homoquinone taken in place of *o*-benzoquinone. 0·2 g. solid homoquinone dissolved in ether was run into 0·5 g. glycine in buffer at p_H 7·0. The reaction mixture was aerated gently for a day, any ammonia escaping in this process being absorbed in $N/20$ H_2SO_4. The whole of the ammonia produced was finally driven over into the absorption bottles by running into the reaction bottle 1·5 g. sodium carbonate in water, the stream of air being then continued for several hours.

The amount of acid neutralised by ammonia produced during the reaction

corresponded to 0·06 g. homoquinone, *i.e.* 30 % of the homoquinone used, the reaction

$$CH_3.C_6H_3O_2 + NH_2.CH_2.COOH + H_2O = NH_3 + CO_2 + H.CHO + CH_3.C_6H_5O_2$$

being assumed. Another experiment gave 34 %.

The homoquinone does not, therefore, yield the equivalent of ammonia. This may be due partly to combination of the ammonia and formaldehyde to give hexamethylenetetramine, and partly to other reactions which lead to the production of highly coloured substances, since during aeration the liquid in the reaction bottle became deeply coloured, being blue by transmitted, red by reflected light. It was similar to a liquid obtained by the action of tyrosinase on *p*-cresol in presence of glycine (Chodat's reaction). Both on addition of alkali became reddish, and on addition of acid blue.

Measurements of oxygen absorption.

In most cases colour changes in the solution, and in some cases the formation of an anilinoquinone in presence of aniline, serve to determine whether a given phenol is oxidised by tyrosinase or not. In certain cases recourse was had to a microrespirometer of the Barcroft type, in which carbon dioxide was absorbed. The following pairs of mixtures were shaken side by side. Respirometer I. Left: the phenol used; phosphate buffer; tyrosinase. Right: buffer; tyrosinase. Respirometer II. Left: the phenol used; buffer. Right: buffer alone. Any autoxidation taking place could thus be detected. At the commencement of each experiment, the apparatus was equilibrated by shaking for 5 minutes with the taps open; the results obtained are therefore minimum values, since in some cases a small amount of oxygen would be absorbed during equilibration. The quantity of phenol used was 1 or 2 mg. Solid phenylurethane or thymol was added to each flask as antiseptic, the enzyme used being freed from chloroform *in vacuo*. The activity of the enzyme at the end of the experiment was tested by addition of *p*-cresol to the left flask of Respirometer I.

The amounts of oxygen absorbed by phenols attacked by tyrosinase were measured in this way, and calculated in terms of atoms of oxygen per molecule of phenol, some indication being thus obtained of the extent of oxidation taking place. The following substances were examined in the respirometer,

Substrate	p_H	Atoms of oxygen absorbed per molecule of substrate
Phenol	7·6	3·4 +
o-Cresol	7·6	Nil
m-Cresol	7·0	2·9
p-Cresol	7·6	2·6
,, ...	7·8	3 } Robinson and
Catechol	7·8	2 } McCance
Resorcinol	7·0	Nil
Quinol	6·0	3·0
Pyrogallol	6·0	1·5
m-Methoxyhydroxybenzene (slightly impure)	7·0	2 +
p-Methoxyhydroxybenzene	7·0	3·0

with the results stated. Two complementary results obtained by Robinson and McCance [1925] are included.

p-Aminophenol hydrochloride, brought to p_H 6·0 with NaOH, and buffered, showed autoxidation, but a definite increase in oxygen absorption in presence of tyrosinase. Thymol was also tested, its absorption being nil.

The figure 1·5 for pyrogallol was obtained after deduction of slight absorption due to autoxidation. The absorption of three atoms of oxygen per two molecules of pyrogallol corresponds to the formation of purpurogallin.

The experiments of Robinson and McCance [1925], in which slight oxygen absorption was obtained with resorcinol in presence of tyrosinase and a delayed but considerable absorption when glycine was also present, were repeated, but no confirmation of their results was obtained. In most cases the enzyme was inactivated by resorcinol after a few days, but in a case in which it remained active no absorption was observed.

Catechol, *m*- and *p*-cresol, *m*- and *p*-methoxyhydroxybenzene all show an absorption per molecule of one atom of oxygen more than that corresponding to formation of *o*-quinone. Phenol shows an even greater absorption. This appears to indicate that some further reaction involving oxidation occurs. In absence of some substance with which they combine, the *o*-quinones appear to undergo further change almost at once. They are found merely in traces (as judged by the aniline reaction) when the action of the enzyme on the phenol has been allowed to go to completion.

Summary.

1. Anilino-*o*-quinones produced by the action of tyrosinase on several phenols in presence of aniline have been isolated. The substances obtained from phenol and catechol were identical. The substances obtained from *m*-cresol, *p*-cresol, and homocatechol were also identical. This indicates clearly that *o*-quinones are produced by the action of tyrosinase on these phenols.

2. Peroxidase and hydrogen peroxide, acting on catechol or homocatechol, also produce *o*-quinones.

3. Further evidence indicating the formation of *o*-quinones consists in the isolation of a sulphinic acid derivative of *o*-benzoquinone by the action of tyrosinase on catechol in presence of benzenesulphinic acid.

4. Homoquinone deaminates glycine in the same manner as does *o*-benzoquinone.

5. *m*- and *p*-Methoxyhydroxybenzene were the only phenols, in addition to those mentioned above, which were found to give rise to anilinoquinones.

6. The oxygen absorbed during the oxidation by tyrosinase of several phenols has been measured, indications being obtained with those phenols giving rise to anilinoquinones that, in absence of aniline, some further reaction involving oxidation occurs beyond formation of the *o*-quinone.

7. The "direct oxidase" theory of Bach and Chodat is criticised in the light of the above results, and a new classification of oxidases into aerobic and anaerobic oxidases is suggested.

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CLXXXI. DISSOCIATION CONSTANTS OF CYSTINE, CYSTEINE, THIOGLYCOLLIC ACID AND α-THIOLACTIC ACID.

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Mathews and Walker [1909] and, in greater detail, Dixon and Tunnicliffe [1923] have demonstrated the great significance of the hydrogen ion activity in relation to the rates of oxidation and of autoxidation of a number of thio-acids. To assist the analysis of these results and of certain observations of our own it became desirable to know the dissociation constants of cysteine, thioglycollic acid and thiolactic acid. It was thought worth while at the same time to determine the corresponding constants of cystine in order that a gap in the electrochemical data on the natural amino-acids might be filled.

With respect to cysteine, thioglycollic acid and thiolactic acid no difficulty was encountered in obtaining reproducible hydrogen electrode titration curves from which the several constants could be deduced. The method was, however, not available in the case of cystine as this substance suffers reduction at the hydrogen electrode surface with consequent depolarisation of the electrode. The quinhydrone electrode suggested itself as an alternative since its potential is positive to that which, on chemical and other grounds, may reasonably be attributed to solutions of cystine. This electrode, indeed, proved satisfactory within its limitations but was rejected because it does not permit the extension of a titration to the low concentrations of hydrogen ions at which stages in the dissociation of cystine occur.

An oxidation-reduction electrode was sought, therefore, which would be available over the whole significant p_H range and whose equilibrium would not be disturbed by the presence of cystine. The methylene blue-methylene white electrode fulfilled these requirements.

Experimental.

The titration vessel used for the hydrogen electrode titrations was similar to that employed by Clark and Cohen [1923] for oxidation-reduction titrations. This carried a pair of palladium-coated, gold-plated platinum electrodes and a supply tube for hydrogen gas. A saturated calomel electrode and a

saturated potassium chloride bridge completed the cell, which was immersed in a kerosene thermostat at 30°. The E.M.F. was determined potentiometrically against a standard Weston cell. At no significant points on the titration curves did the two electrodes differ by more than 0·1–0·3 mv. In the type of cell employed the hydrogen stream is delivered through the solution but not directly over the electrode surfaces. Consequently the initial attainment of equilibrium at the electrode occupies some time after the introduction of the solution to be titrated: 20–30 minutes was found adequate. Subsequent adjustments to additions of titrating agent are rapid, and true equilibrium values can be determined in 2–3 minutes after each addition.

For our own purposes we required to know the p_H of approximately 0·05 molar solutions of the sulphydryl compounds neutralised to varying degrees with 0·5 molar CO_2-free sodium hydroxide. For this reason the electrometric titrations were performed with solutions of similar strength. It was appreciated that the constants derived from observations on solutions of such ionic strength are subject to a significant correction for activity. No such correction has been applied because, in the case of the two ampholytes, its calculation is uncertain, and because most of the data in the literature on the constants of the natural amino-acids are uncorrected values (K') referable to solutions of about 0·1 molar strength. Some indication of the magnitude of the activity correction was obtained by repeating the titrations on 0·01–0·02 molar solutions. A further advantage in working at fairly high concentration is the more extensive data which can be obtained on the constants functioning at the extremes of the p_H scale.

Cystine was prepared by the acid hydrolysis of hair and recrystallised several times. From this, cysteine hydrochloride was obtained by reduction with zinc and hydrochloric acid, precautions being taken against re-oxidation during crystallisation. Thioglycollic acid (B.P. 109–110°/16 mm.) was prepared from monochloroacetic acid and sodium disulphide followed by reduction of the dithiodiglycollic acid with zinc and hydrochloric acid. α-Thiolactic acid (B.P. 107–110°/17 mm.) was prepared by the method of Lovén [1884] from α-chloropropionic acid. These acids were redistilled at low pressure in nitrogen.

The titration curves are assembled in Fig. 1, in which the "corrected equivalents of base" [Simms, 1926] are plotted against the observed p_H values.

Table I, which is later explained, records some of the data from which the curve for cysteine has been constructed and serves to exhibit the order of consistency attained throughout the work.

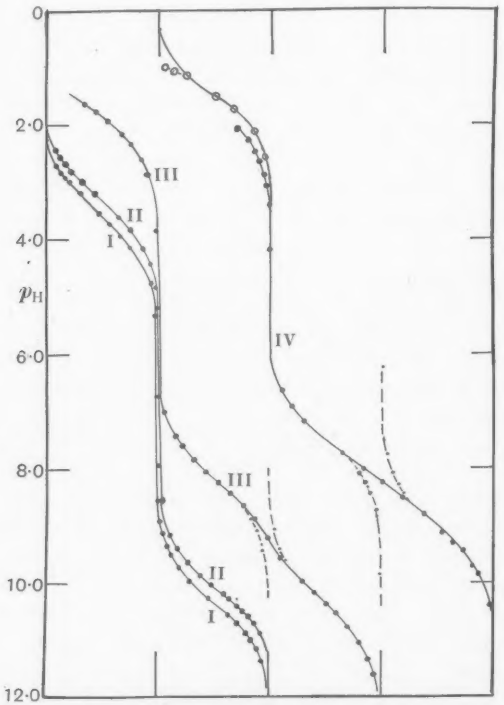
It is apparent, in respect of thioglycollic acid and thiolactic acid, that the two constants differ so greatly in magnitude that the curve may be treated as that of a mixture of two monobasic acids, whose constants are derived from the relation

$$p_{K'} = p_H - \log \frac{a}{1-a}.$$

Table I. *Titration of* 0·1 *M cysteine hydrochloride with M sodium hydroxide.* 30°.

$p_{K_1'}=1{\cdot}86$ $p_{K_2'}=8{\cdot}14$ $p_{K_3'}=10{\cdot}34$

p_H	a_1	a_2	a_3	Corrected equivalents of base: Calculated	Found
1·626	0·368	—	—	0·368	0·343
1·675	0·395	—	—	0·395	0·379
1·841	0·489	—	—	0·489	0·485
2·030	0·597	—	—	0·597	0·596
2·151	0·661	—	—	0·661	0·675
2·340	0·769	—	—	0·769	0·755
2·860	0·909	—	—	0·909	0·907
7·000		0·0676	0·0000	1·068	1·081
7·607	One equivalent complete	0·2266	0·0004	1·227	1·234
8·046		0·4450	0·0023	1·450	1·447
8·422		0·6516	0·0079	1·667	1·660
8·887		0·8236	0·0290	1·882	1·872
9·200		0·8624	0·0625	1·988	1·979
9·545		0·8334	0·1336	2·101	2·106
9·965		0·6962	0·2935	2·283	2·298
10·160		0·5987	0·3955	2·390	2·404
10·520		0·3971	0·6011	2·599	2·610
10·756		0·2771	0·7219	2·721	2·712
11·035		0·1679	0·8320	2·832	2·798
11·595		0·0526	0·9475	2·948	2·934



Corrected equivalents of base.

Fig. 1.

Curve I=α-Thiolactic acid. Curve II=Thioglycollic acid. Curve III=Cysteine.
Curve IV=Cystine {●—●—● *M*/50 M. blue electrode. ○—○—○ *M*/10 Quinhydrone electrode.

The case of cysteine is more complex. To avoid the necessity for discriminating between acidic and basic constants in the analysis it is of assistance to regard the kation of the hydrochloride as an acid dissociating three hydrogen ions. The constants K_1', K_2', K_3' refer to the successive steps without any present implication as to the specific group which determines the value of each constant.

$$R\begin{cases}NH_3^+\\COOH\\SH\end{cases} \rightarrow R\begin{cases}NH_2^+\\COO^-\\SH\end{cases} \rightarrow R\begin{cases}NH_3^+\\COO^-\\S^-\end{cases} \rightarrow R\begin{cases}NH_2\\COO^-\\S^-\end{cases}$$

It is clear from the curve that the value of K_1' is so much greater than those of K_2' and K_3' that it may be derived separately. The influences of K_2' and K_3', however, significantly overlap. This region of the curve was therefore treated as that of a dibasic acid. Graphic approximations to the values of $p_{K_2'}$ and $p_{K_3'}$ were made and the values of α_2 (primary ion) and α_3 (secondary ion) were calculated from the equations

$$\alpha_2 = \frac{1}{1+\frac{[H^+]}{K_3'}+\frac{K_2'}{[H^+]}}$$

$$\alpha_3 = \frac{1}{1+\frac{[H^+]}{K_3'}+\frac{[H^+]^2}{K_2'K_3'}},$$

which are derived from the application of the mass law [Michaelis, 1921]. Thence were calculated the equivalents of base $(1+\alpha_2+2\alpha_3)$ required to bring to the observed p_H values a mixture of three acids having the given constants. These values are compared with the corrected equivalents of base derived from the titration readings. Minor modifications of the graphic values of $p_{K'}$ were then made to improve the alignment of the above data[1].

Methylene blue-methylene white electrode.

Clark, Cohen and Gibbs [1925] have found that the electrode potential of a given ratio of the oxidised and reduced forms of this system varied not only with different samples of the dye but also with the total concentration present. Fortunately we were able to overcome these difficulties through the gift of a specimen of Sample *F* of the methylene blue for which Clark, Cohen and Gibbs have recorded electrode data. The dye was employed at a similar concentration to that with which they worked (0·0001 *M* total dye). Into a burette, protected from oxygen, was introduced a solution, approximately equimolar, of methylene blue and methylene white. The potential of this mixture at a gold-plated platinum electrode at p_H 3·97 was determined by adding 5 cc. to 35 cc. of 0·057 *M* potassium acid phthalate.

[1] As Simms [1926] has pointed out, these constants are titration constants (G') and are related to the true dissociation constants (K') by the equations

$$K_1' = G_1' + G_2',$$

$$\frac{1}{K_2'} = \frac{1}{G_1'} + \frac{1}{G_2'}.$$

The correction is negligible in the present case.

The potential of the methylene blue-methylene white electrode is related to $[H^+]$ by the following equation [Clark, Cohen and Gibbs, 1925] (which holds acid to p_H 12):

$$E_h = C + 0{\cdot}03006 \log [K_1K_2 [H^+] + K_2 [H^+]^2 + [H^+]^3]$$

where E_h is the potential referred to the normal hydrogen electrode,

$$K_1 = 1{\cdot}4 \times 10^{-6} \text{ and } K_2 = 3{\cdot}0 \times 10^{-5},$$

being dissociation constants of methylene white.

C is a constant given by

$$C = 0{\cdot}533 - 0{\cdot}03006 \log \frac{[\text{methylene white}]}{[\text{methylene blue}]}.$$

For any experimental ratio of the oxidised and reduced forms C can be calculated by determining the potential (E_h) at a known p_H. With this determined the equation may be used to relate observed potentials of the given mixture to the p_H of the solution to which it has been added. The equation is cumbersome, however, and it is convenient that K_1 and K_2 are of such an order that within the range p_H 1–2·5 the relation reduces to $E_h = C - 0{\cdot}09018\ p_H$; and between p_H 7·8–12·00 to

$$E_h = C - 0{\cdot}313 - 0{\cdot}03006\ p_H.$$

In view of the low solubility of cystine the titration was carried out on a 0·02 molar solution containing sufficient standard hydrochloric acid to effect solution. To 35 cc. of this solution were added 5 cc. of the dye mixture and the titration was conducted in purified nitrogen with oxygen-free sodium hydroxide. The excellent agreement between the potentials of two electrodes, their reproducibility, the absence of potential drifts, and the satisfactory analysis to which the data submitted gave assurance that the electrode gave a true measure of the p_H. No precipitation of the cystine was observed at any stage of the titration.

In the case of cystine there are four constants to be determined. By analogy with cysteine and other amino-acids it will be expected that one pair will be active in the acid range and one on the alkaline side of neutrality. The curve shows this to be so and permits each pair of constants to be derived by treating their section of the curve as that of a dibasic acid, as was done in the case of cysteine. As a result it appears that K_1' is too great to be measured. K_2', K_3' and K_4' have been deduced. K_2' was determined also by titration of a 0·1 molar solution of cystine hydrochloride at the quinhydrone electrode. Precipitation of cystine did not occur, even at this concentration, until the titration of the first two equivalents was almost complete.

The third and fourth titration constants lie so close together that they differ slightly from the true constants. Applying the correction of Simms [1926] (footnote, p. 1387) we get for the uncorrected apparent dissociation constants the values in Table II.

Table III is an abbreviation of the data for K_3' and K_4' constructed in the same way as Table I. It serves to show the order of consistency of the methylene blue electrode readings.

Table II. *Apparent dissociation constants (K') at 30°: not corrected for activity.*

	Approx. molar strength	$p_{K_1'}$	$p_{K_2'}$	$p_{K_3'}$	$p_{K_4'}$	K_1'	K_2'	K_3'	K_4'
Thioglycollic acid	0·1	3·4	10·0	—	—	4×10^{-4}	1×10^{-10}	—	—
	0·01	3·5	10·2	—	—	3×10^{-4}	6×10^{-11}	—	—
Thiolactic acid	0·025	3·6	10·3	—	—	$2{\cdot}5 \times 10^{-4}$	5×10^{-11}	—	—
	0·01	3·7	10·3	—	—	$2{\cdot}0 \times 10^{-4}$	5×10^{-11}	—	—
Cysteine	0·1	1·86	8·14	10·34	—	$1{\cdot}4 \times 10^{-2}$	$7{\cdot}3 \times 10^{-9}$	$4{\cdot}6 \times 10^{-11}$	—
	0·02	1·96	8·18	10·28	—	$1{\cdot}1 \times 10^{-2}$	$6{\cdot}6 \times 10^{-9}$	$5{\cdot}3 \times 10^{-11}$	—
Cystine	0·1	<1·0	1·5	—	—	$>1 \times 10^{-1}$	$3{\cdot}2 \times 10^{-2}$	—	—
	0·02	<1·0	1·7	7·48	9·02	$>1 \times 10^{-1}$	$2{\cdot}0 \times 10^{-2}$	$3{\cdot}3 \times 10^{-8}$	$9{\cdot}6 \times 10^{-10}$

Table III. *Titration of 0·02 M cystine hydrochloride with 0·1 M sodium hydroxide.* 30°.

$p_{K_1'} = < 1{\cdot}00$ $p_{K_2'} = 1{\cdot}72$ $p_{K_3'} = 7{\cdot}48$ [$pG_3' = 7{\cdot}50$] $p_{K_4'} = 9{\cdot}02$ [$pG_4' = 9{\cdot}00$]

					Corrected equivalents of base	
p_H	a_1	a_2	a_3	a_4	Calculated	Found
2·26	Assume	0·776	—	—	1·776	1·792
2·47	1 equivalent	0·849	—	—	1·849	1·853
2·63	completed	0·889	—	—	1·889	1·887
2·87		0·935	—	—	1·935	1·935
3·07		0·957	—	—	1·957	1·957
6·62			0·1164	0·0005	2·117	2·117
6·90	2 equivalents completed		0·2005	0·0016	2·204	2·206
7·15			0·2611	0·0035	2·268	2·296
7·44			0·4591	0·0127	2·484	2·474
7·72			0·6039	0·0317	2·667	2·652
8·02			0·7109	0·0745	2·870	2·830
8·25			0·7375	0·1342	3·006	3·010
8·52			0·7008	0·2321	3·165	3·188
8·81			0·5899	0·3810	3·352	3·366
9·12			0·4267	0·5628	3·552	3·542
9·30			0·3316	0·6600	3·652	3·639
9·45			0·2609	0·7225	3·706	3·718
9·71			0·1793	0·8360	3·851	3·804
9·85			0·1412	0·8757	3·893	3·854
10·41			0·0375	0·9627	3·963	3·946

Remarks.

The treatment which has been accorded the dissociation of cysteine and cystine has not distinguished acidic and basic constants. It is still a common custom to refer the first dissociation constant of amino-acids to the basic group and the second to the acid group, *i.e.*, an amino-acid is regarded as both a weak acid and a weak base. On this basis the K_1' here reported for cysteine, and the K_1' and K_2' for cystine would be the hydrolysis constants of the NH_2 groups related to K_b by $K_b = \frac{Kw}{K'}$.

Adams [1916] and Bjerrum [1923] on the other hand have adopted the more plausible view that the constant active at an acid reaction is the acidic constant and that functioning at low [H^+] is referable to the NH_2 group. In the case of cystine, therefore, K_1' and K_2' relate to the symmetrical COOH groups and K_3' and K_4' become the hydrolysis constants of the NH_2 groups. The enhanced values of the acid constants and the diminution in strength of

the basic groups in comparison with other amino-acids is to be remarked. In the case of cysteine we may give K_1' to the carboxyl group but there is little to distinguish the influence of the sulphydryl- and amino-groups in the two stages of dissociation in the alkaline range. Comparison with the simple thio-acids which have been titrated would suggest that K_3' of cysteine is determined by the sulphydryl group. On the other hand the – SH group in cysteine is further removed from the COOH than in thioglycollic and in thiolactic acids, and should therefore be a stronger group. Moreover K_3' approximates much more closely to the values of the NH_2 constant of related amino-acids (*e.g.* alanine, serine) than does K_2'. It is probable that a plausible argument could be made for either allocation of K_2' and K_3' in cysteine. A decision seems premature and in any case can only be qualitatively true in view of the fact that in cases where two constants are of similar magnitude both groups involved are concerned in determining the value of each constant.

Acknowledgment is made to the Medical Research Council for a grant from which the expenses of this work were defrayed.

One of us (B. C. J. G. K.) is further indebted to the Department of Scientific and Industrial Research for a grant which enabled him to take part in this work.

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CLXXXII. STUDIES IN THE METABOLISM OF TISSUES GROWING *IN VITRO*.

II. EFFECT OF GLUCOSE UPON THE AMMONIA AND UREA PRODUCTION OF KIDNEY TISSUE.

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(*Received October 31st, 1927.*)

In the first paper of this series [Holmes and Watchorn, 1927] we described the formation of ammonia and urea by the tissue of embryonic rat kidneys when this was growing *in vitro*. The formation of these substances occurred only when actual growth took place, and we were inclined to believe that it indicated the combustion of nitrogenous substances to supply the energy necessary for growth. If this were the case, the addition of glucose, *i.e.* the supplying of another and probably more available source of energy, might be expected to lessen the production of ammonia and urea during the growth of the tissue.

These experiments were undertaken to discover whether such an effect is produced by adding glucose to the culture medium.

Technique.

This has been essentially the same as that previously described [Holmes and Watchorn, 1927]. Some modifications of Stanford's ammonia method [1923] have been introduced. We have used much hotter water for surrounding the distilling flask—namely about 60°. If the apparatus has just been well evacuated, this enables brisk distillation to proceed for 12 minutes, or often longer, without the apparatus being attached to the filter pump. It should, however, be connected to the pump for two or three minutes before finally discontinuing distillation, otherwise a slightly low result is apt to be obtained. When letting down the vacuum, air should be admitted through a sulphuric acid wash-bottle. At no period during distillation do we pass air through the apparatus. If cooler water is used, as described by Stanford, attachment to the filter pump is necessary after about 3 minutes, and under these conditions we have found that usually only 90 % of the ammonia distils over. We have assured ourselves that, at any rate for our solutions, the hotter water is no disadvantage.

Glucose determinations have been made by Hagedorn and Jensen's [1923] method. Hydrogen ion concentration has been measured by aid of the B.D.H. capillator, using bromothymol blue as indicator, which completely covers our range.

Experimental.

Pannett and Compton's [1924] Ringer solution was used for diluting the embryo extract. Varying amounts of glucose have been added at different times. As we previously showed that no ammonia or urea was formed by resting tissue (*i.e.* tissue which was living, but was floating freely in the medium and thus unable to grow) after 48 hours' incubation at 37° we have frequently discarded the elaborate system of controls formerly used, and have simply taken the resting tissue preparations as controls for the growing tissues. Preliminary experiments with resting tissues have shown that when kidneys from the same litter are used, the addition of glucose to an otherwise similar medium does not affect the non-occurrence of ammonia or urea formation by non-growing, non-autolysing tissue (Table I).

Table I.

	Mg NH_3-N		Mg urea-+NH_3-N	
Exp.	Without glucose	*With glucose	Without glucose	*With glucose
20	0·036	0·034	0·060	0·061
22	0·032	0·032	0·062	0·061
29	0·048	(i) 0·046 (ii) 0·042	0·084	(i) 0·087 (ii) 0·083
30	—	—	0·056	(i) 0·053 (ii) 0·055
32	0·044	0·046	0·114	0·119
34	0·027	0·026	0·082	0·075

* The original concentration of glucose in these cases is from 0·07 to 0·09 %.

Having established this fact, it was then possible to use resting tissue preparations containing glucose as controls for growing tissue.

The addition of glucose gave rise to a marked alteration in the metabolism of the growing tissue, in that it caused a very definite inhibition of ammonia and urea formation. Unfortunately, as the number of embryos in a litter is sometimes small, it has not always been possible to include preparations of growing tissues in plain and in glucose media in the same series. This is not a very serious drawback, as we have previously obtained a large number of experimental results showing that ammonia and urea are formed when the tissue grows in the absence of glucose. In the presence of glucose the formation of these substances is, as is shown by comparison with the resting controls, frequently completely inhibited. Under these circumstances, when the results obtained are exactly similar in growing preparations and in controls, it is necessary to be certain that growth has actually taken place. Even if tissue without glucose is included in the same series, and is known to have grown well, there is obviously no certainty that the glucose-fed tissue has also grown, as the presence of the sugar may either inhibit or accelerate growth. Except in the early experiments, we have therefore included in each series one or more small control dishes containing small amounts of tissue growing in the same medium, which could be used for staining and microscopical

examination. These revealed the fact that very small amounts of glucose in the medium did not inhibit, but if anything aided growth. In some cases better differentiation seemed to take place in the presence of glucose than in its absence. However, on one or two occasions when a concentration of over 0·2 % was present, inhibition of growth and even death of the tissue apparently took place[1]. This concentration is, of course, remarkably low compared with figures given for possible glucose concentrations by other observers [Lewis, 1922; Willmer, 1927], who find 1 % and even 2 % solutions suitable for growth. This difference may be due to the fact that we have used mammalian tissue, whereas other observers have dealt with chick tissue. The amount of glucose in the egg is fairly large at some stages of its development, and it is possible that the tissues of the embryo are exposed to relatively high concentrations. Indeed, the blood-sugar in embryo chicken blood is about 0·2–0·3 % at some stages of development [Hanan, 1925]. Willmer [1927] states that 0·2 % would be the best strength for the *in vitro* growth of chick intestine if it could be constantly maintained. In the case of embryo rat kidney we find that concentrations approaching mammalian blood-sugar level (*i.e.* 0·07 to 0·11 %) are more suitable.

Figures for tissues growing in a glucose medium are given in Table II. The concentration of glucose originally present was from 0·07 to 0·11 %.

Table II.

	Control		Growing tissue	
Exp.	NH_3-N	NH_3- + Urea-N	NH_3-N	NH_3- + Urea-N
20	0·036	0·060	0·039	0·064
22	0·032	0·062	0·033	0·052
			0·024	0·051
29	0·048	0·084	0·042	0·069
			0·039	0·069
30	0·028	0·066	0·028	0·067
33	0·046	0·120	0·046	0·108
34	0·026	0·075	0·027	0·055
			0·029	0·072
35	0·028	0·055	0·026	0·057
36	—	0·079	—	0·080

It will be seen that, in some experiments, the growing tissue has the same figure for ammonia and urea as its control, but that in others (Exps. 22, 29, 33 and 34) the total ammonia- and urea-nitrogen is actually less in the growing preparation than in the resting tissue. It is perhaps significant that these were all flasks in which good growth had taken place. When tissues grown in plain and in glucose media are included in the same series (that is to say, when all kidneys used are taken from the same litter, and the media made from the same embryo extract) then the results are even more striking (Table III).

[1] The glucose solutions used in these cases had been autoclaved, and their toxicity may be due to this.

Table III.

	Resting control		Tissue growing in absence of glucose		Tissue growing in presence of glucose		Increase in NH_3- + Urea-N	
Exp.	NH_3-N	NH_3- + Urea-N	NH_3-N	NH_3- + Urea-N	NH_3-N	NH_3- + Urea-N	Without glucose	With glucose
20	0·036	0·060	0·050	0·079	0·039	0·064	0·019	Trace
			0·075	0·075			0·015	
28	0·045	0·076	0·060	0·105	—	0·092	0·029	0·016
30	0·028	0·066	0·050	0·115	0·028	0·067	0·049	Nil
35	0·028	0·055	0·027	0·084	0·026	0·057	0·029	Nil
				0·085			0·030	

The concentrations of glucose originally present vary from 0·07 to 0·11 %.

In Exp. 30, for instance, which is shown in this table, the growth in the glucose medium was particularly good, yet the content of ammonia- and urea-nitrogen showed no rise, whereas in the plain medium the growth of the tissue caused a 74 % increase in the amount of ammonia and urea present. Exp. 35 in Table III gives another very definite illustration of the effect of the addition of glucose.

We had hoped when this work was first begun that we might find a striking quantitative difference in the carbohydrate metabolism of the floating (resting) and the growing tissues. Up to the present we have only attempted to estimate the disappearance of glucose, and the results have been somewhat disappointing, as there is often no such difference found. As might have been expected from the work of Warburg and others, the resting kidney tissue itself is active in breaking down glucose; thus, anything which delays the commencement of growth or diminishes its extent must tend to obscure any quantitative differences between the resting and the growing preparations. Again, estimations showing the disappearance of glucose from the medium give no indication of the manner in which the sugar may be utilised. It is possible, for instance, that the breakdown of the glucose may be far more complete, and the energy obtained therefore far greater in amount, in some preparations than in others. It may even happen that further breakdown of glucose occurs after the flasks are removed from the incubator, and we have taken no special precautions to prevent this.

Although in many cases the resting tissue has caused as much glucose to disappear as the growing tissue, it has been possible in experiments where very good growth has occurred to demonstrate an extra glucose breakdown on the part of the growing tissue. Thus, in Exp. 30, the original amount of glucose present in each flask was 1·5 mg., the amount remaining in the control preparation 0·9 mg., and the amount remaining in the growing preparation only 0·7 and 0·4 mg. The glucose estimations are carried out on small portions of the filtrates in which the ammonia and urea values are determined. A control flask is kept at − 2° for the duration of the experiment and is used for estimation of the amount of glucose originally present; we

have usually found that about one-third of this amount is broken down by the resting tissue.

p_H determinations have also been carried out on the filtrates. These have been found to give a rough check on the figures we obtain for glucose breakdown, and are more generally useful in determining the optimum hydrogen ion concentration for tissues growing under our particular conditions.

Discussion.

We had previously shown that kidney tissue which was growing in a plain embryo extract always formed considerable amounts of urea and ammonia, though resting (floating) tissue under identical conditions did not.

We have now found that, in the presence of glucose, very good growth may occur without any production of urea and ammonia by the growing tissue.

It seems probable, therefore, that, in the absence of glucose, nitrogenous substances are broken down to provide the energy necessary for the growth of the tissue, thus giving rise to the ammonia and urea which appear. When glucose is present the necessary energy may be obtained instead from the breakdown of glucose.

In a great many cases we have obtained very good growth in glucose media without any increase (sometimes even a slight decrease) in the ammonia- and urea-nitrogen. Sometimes a slight but definitely lessened production of these substances is found, while on one occasion the increase in ammonia- and urea-nitrogen seemed to be unaffected by the presence of the glucose, although it is probable that in this case the extent of the growth was greater in the glucose medium than in the plain.

We have no explanation for these variations, which may be due to differences in the age of the embryos, alterations in the glucose during sterilising, or varying proportions in the media of glucose and the nitrogenous substances which are broken down by the tissue. There is no doubt, however, that we have obtained very definite evidence of the so-called "protein-sparing" action of glucose.

Raistrick [1921], dealing with the effect of glycerol upon the breakdown of tryptophan by bacteria, claims that glycerol cannot be said to "spare" the amino-acid, since, although less ammonia is formed when the glycerol is present, a larger amount of the nitrogen from the tryptophan is built up into the bacterial tissues. Raistrick considers that the effect of the addition of glycerol is to produce a more suitable carbon/nitrogen ratio for bacterial growth. Even in this case, however, there was (this was pointed out to us by Miss M. Stephenson) more tryptophan left untouched at the end of the experiment when the bacteria were grown in the glycerol medium, than when they were grown in a medium containing tryptophan alone, so that some actual sparing of the amino-acid did occur. We are inclined to believe that in our case the glucose has much this effect, since, although the oxidative

breakdown of nitrogenous substances (or that part of it which is manifested by the appearance of ammonia and urea) is largely prevented, the growth is probably greater in extent, and therefore more nitrogen must be removed from the medium for purposes of synthesis.

Lewis [1922], Willmer [1927] and others have employed glucose in culture media, and consider it useful in maintaining growth. Lewis, in fact, finds that it much prolongs the life and activity of cells which are allowed to grow for some time in the same drop of culture medium. It will be seen from consideration of the facts set forth in this paper that this prolongation of life and the power of growth may be due to the addition of extra carbohydrate fuel, or to the prevention of the accumulation of ammonia and possibly other products of protein breakdown. It is highly probable that unduly high concentrations of ammonia are very toxic for tissue cells. This is in fact indicated by the work of Krontovski and Radzimovska [1922] who showed that exposure of tissues for half an hour to Michaelis' ammonia standards inhibited the subsequent growth of the cells far more than the alkalinity of the solutions warranted.

In a previous paper [Holmes and Watchorn, 1927] we discussed the apparent contradiction between our results and those of Bollman, Mann and Magath [1924] who found that the liver is the only organ in the body which produces urea. The growing embryonic kidney can, however, produce ammonia, urea or both together. On the other hand, the presence of a little glucose frequently prevents the appearance of these substances, and in the experiments of Bollman, Mann and Magath the blood-sugar was kept at a normal, even slightly supernormal, level. Thus it is not possible to state at present that the formation of urea occurs only when the kidney tissue is growing, and not in the adult, functioning kidney. The origin of the ammonia, which is excreted by functioning kidney when the glomerular filtrate is acid, is not known.

What has been commonly spoken of as the "protein-sparing" power of carbohydrates is in the intact animal a complex phenomenon. It seems of interest to have demonstrated directly such an action in the case of an isolated tissue removed from the immediate influence of insulin and other hormones which affect the metabolism in the body of the whole animal.

Summary.

(1) Addition of glucose to the medium makes no difference to the non-occurrence of ammonia and urea production by non-growing, but surviving embryonic rat-kidney tissue.

(2) Addition of glucose to embryonic rat-kidney tissue growing *in vitro* generally inhibits the production of ammonia and urea which would otherwise take place, and frequently actually causes a reduction in the amount of these substances initially present.

We should like to thank Sir F. G. Hopkins for his interest in this research.

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CLXXXIII. THE EFFECT OF ADRENALINE ON KETOSIS IN PHLORIDZINISED AND NORMAL RATS.

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(*Received October 31st, 1927.*)

In the course of metabolic experiments on phloridzinised rats it was observed that doses of adrenaline increased the excretion of acetone bodies. This paper gives an account of some experiments undertaken in an attempt to investigate this increase and a discussion of its significance in relation to other published observations on the action of adrenaline.

Rats were phloridzinised by Coolen's method, receiving 50 mg. phloridzin in oil every day or every two days. Urine was collected in 24-hour samples in the usual way. Acetone bodies were estimated by the method used by Wigglesworth [1924]. A satisfactory diet for the fully phloridzinised rat had been found in previous work to consist of caseinogen 75 parts, fat 20, salts 5, agar-agar 5. The salt mixture was that employed by Wigglesworth ($Ca_3(PO_4)_2$ 15, Na_2HPO_4 10, KH_2PO_4 27·5, NaCl 10, KCl 5). On this diet the animals remained in fairly good condition even when completely phloridzinised for periods up to 3 weeks. The daily excretion of acetone bodies was about 4–8 mg. in normal rats, rising slightly on phloridzinisation to 5–15 mg.

The subcutaneous administration of adrenaline in doses of 0·5 mg. to phloridzinised rats caused a large rise in the excretion of acetone bodies. Figs. 1 and 2 show the effect in two animals: in (1) the total acetone bodies rose after administering adrenaline to 63 mg. and in (2) to 62 and 82 mg.; in each case the excretion fell to the normal level next day. In non-phloridzinised rats on the above diet or on a standard diet containing carbohydrates no rise in acetone body excretion was observed after the administration of adrenaline (see Fig. 1, days 1–5, and Fig. 3). In non-phloridzinised rats on a diet of fat with 5 % salts, adrenaline produced a small rise, the effect being more marked in young animals in which there was a greater ketosis. Thus Fig. 4 for an old rat shows a rise from 2·5 mg. acetone bodies to 5·7 mg. after adrenaline, and Fig. 5 for a young animal shows a larger initial ketosis of about 11 mg. gradually falling to 2 mg., with peaks of 23·9, 24·3, and 14·3 mg. after adrenaline. It was found that rats on this fat diet could not survive phloridzinisation for more than a day or two.

In view of the possibility that this rise in ketosis might be due to the acidosis produced by adrenaline, rats on the protein-fat diet + 5 % $NaHCO_3$ were phloridzinised and given adrenaline. The excretion of acetone bodies was at a higher level in these animals, as was expected from the finding of Wigglesworth [1924] that alkali produces ketosis, but adrenaline still caused a large rise comparable with that obtained on the protein-fat diet without alkali. Fig. 6 illustrates this, the rise being from about 20 mg. to 68 mg., falling to the original level next day.

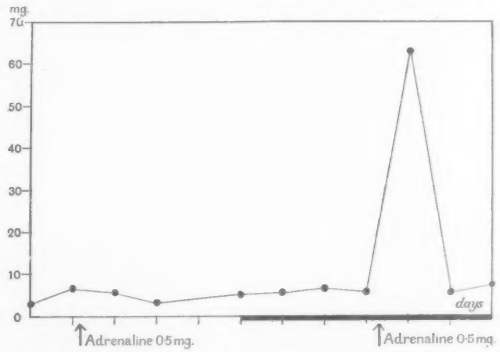

Fig. 1. Abscissa: days. Ordinate: excretion in mg. per day of acetone bodies (acetone +acetoacetic acid+β-hydroxybutyric acid). Rat of about 165 g., put on protein-fat diet 4 days before beginning of experiment. Thickened portion of base line indicates period of phloridzinisation.

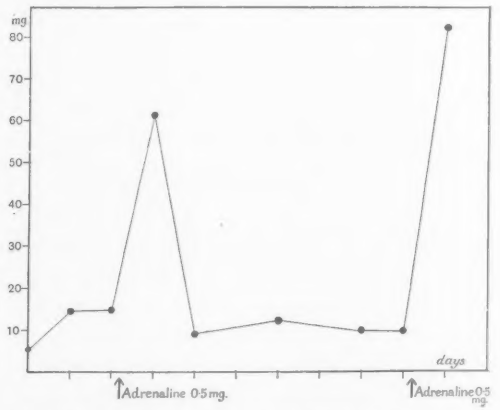

Fig. 2. Abscissa and ordinate as in Fig. 1. Rat of about 200 g., put on protein-fat diet one day before beginning of experiment; phloridzinised throughout experiment.

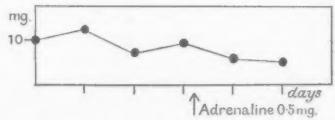

Fig. 3. Abscissa and ordinate as in Fig. 1. Rat of about 190 g., put on standard complete diet 9 days before beginning of experiment.

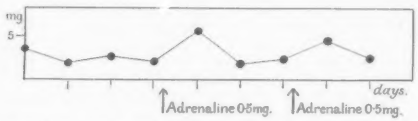

Fig. 4. Abscissa and ordinate as in Fig. 1. Rat of about 195 g., put on fat diet 2 days before beginning of experiment.

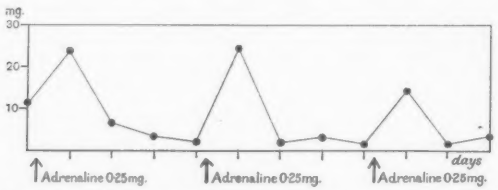

Fig. 5. Abscissa and ordinate as in Fig. 1. Young rat of about 130 g., put on fat diet 3 days before beginning of experiment.

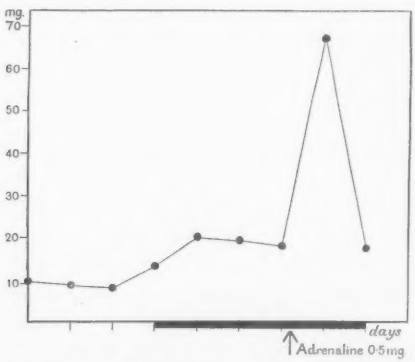

Fig. 6. Abscissa and ordinate as in Fig. 1. Rat of about 195 g., put on protein-fat diet + 5% $NaHCO_3$ 3 days before beginning of experiment. Thickened portion of base-line indicates period of phloridzinisation.

Discussion.

In the experiments reported above we found a large rise in the excretion of acetone bodies with a quick return to the previous level. Since the effects of adrenaline only last for a few hours, this striking increase in the excretion of acetone bodies is no doubt even more intense than our figures for the

24 hour periods show. It seems that adrenaline may have a specific effect on fat metabolism, possibly in hastening β-oxidation and thereby the production of four-carbon residues. The increase in excretion of acetone bodies was only observed in cases where there was already a tendency to ketosis. If adrenaline exerts a specific stimulus to fat metabolism under normal conditions of diet, the amount of acetone bodies produced is not more than can be completely oxidised by the body.

That adrenaline causes a rise in metabolism is a well established fact; heat production and oxygen consumption are both increased. Lusk and Riche [1914], Palladin and Tichwinskaja [1925], and other observers have found no increase in protein metabolism. Increased combustion of carbohydrate might be suspected in view of adrenaline hyperglycaemia. But Boothby and Sandiford [1923] have pointed out that "the calorigenic action of adrenalin cannot be due simply to the presence of excess carbohydrate," for ingestion of sugar resulting in a blood-sugar much in excess of that in adrenaline hyperglycaemia gave only a small rise in heat production; an experiment on a diabetic showed no increase in metabolism after ingestion of 100 g. glucose but a marked increase after adrenaline with only a slight rise in blood-sugar. Stricker and Bouckaert [1924] injected glucose slowly into dogs till the blood-sugar was up to 0·3 % and found a slight fall in heat production, while adrenaline caused a sharp rise for a rather smaller hyperglycaemia. Thus the increase in metabolism after adrenaline cannot be wholly explained by the increase in free glucose.

The rise in the R.Q. after adrenaline found by most workers appears at first sight to rule out the possibility of an increase in fat metabolism. But a careful investigation of the figures given by Boothby and Sandiford [1923] and Krantz and Means [1926] shows that this rise is small and seems to precede the peak of heat production, pulse rate, blood pressure, etc. The apparent rise in R.Q. may be due to a washing out of CO_2 from the blood consequent on the decrease of alkaline reserve caused by adrenaline; thus Bornstein [1921] found that adrenaline caused increased lung ventilation and suggests that the temporary rise in R.Q. is due to loss of CO_2 and not necessarily to a larger combustion of carbohydrate. He observed that after the preliminary rise the R.Q. usually fell below normal.

The experiments of Hubbard and Wright [1921] on men and those of Raab [1926] on dogs may be quoted as more direct evidence that adrenaline affects fat and ketone body metabolism; these workers found an increase in acetone bodies in the blood after the administration of adrenaline, while Raab and also Fleisch [1926] observed a fall in blood-fat.

A temporary acidosis after adrenaline has been observed by Foa and Gatin-Gruzewska [1905], Peters and Geyelin [1917], Elias and Sammartino [1921], and others. In our experiments in which adrenaline was given to phloridzinised rats on a diet containing sufficient $NaHCO_3$ to produce an alkalosis, the rise in acetone bodies after adrenaline was comparable with

that obtained without the addition of bicarbonate to the diet. Also Wigglesworth [1924] found that an acidosis caused by NH_4Cl or $NH_4H_2PO_4$ reduced ketosis due to a fat diet or to alkali sometimes to a level below that found in rats under normal conditions. From these considerations it would seem that the acidosis is not responsible for the rise in excretion of acetone bodies after adrenaline.

In a recent paper Levine and Smith [1927] report that fasting for periods of from 4 to 7 days produced no appreciable ketosis in the rat, and that the output of acetone bodies during starvation was similar to that of animals consuming various diets. Thus in our experiments a lack of food intake during the adrenaline period cannot be responsible for the increased ketosis.

We suggest that the large rise in excretion of acetone bodies caused by adrenaline in phloridzinised or fat-fed rats may be due to a temporary intense stimulus to the oxidation of fat, the production of acetone bodies being increased beyond the capacity of the body to oxidise these substances.

Summary.

1. The administration of adrenaline to phloridzinised rats on a protein-fat diet causes a very large temporary increase in the excretion of acetone bodies.

2. In non-phloridzinised rats on the protein-fat diet or on a standard diet containing carbohydrates, no rise in acetone body excretion is observed after the administration of adrenaline.

3. Adrenaline causes an increase in the ketosis of rats on a fat diet.

4. Addition of $NaHCO_3$ to the protein-fat diet does not prevent this effect of adrenaline.

5. It is suggested that the large rise in excretion of acetone bodies caused by adrenaline may be due to a temporary intense stimulus to the oxidation of fat.

We wish to thank Sir F. G. Hopkins and Mr J. B. S. Haldane for their interest in this work, which was done during the tenure by M. D. A. of a Fellowship at Newnham College and a part-time grant from the Medical Research Council.

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Note added December 3rd, 1927. While this paper was in the press, Burn and Ling (*J. Physiol.* **64**, xxii) published work reporting that adrenaline diminishes or inhibits ketonuria in rats on a diet of fat. The conditions were not the same as in our experiments: the doses of adrenaline were smaller and more frequent, and were given during the larger initial ketosis of the 2nd and 3rd days on the fat diet, not as in our experiments, after the animals had adapted themselves to the diet.

CLXXXIV. THE AUTOCATALYTIC OXIDATION OF SULPHYDRYL COMPOUNDS.

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In previous papers by Warburg and Sakuma [1923], by Toda [1926] and by the author [Harrison, 1924, 1927], it was shown that minute traces of certain catalytic metals such as iron are capable of producing a great acceleration in both the aerobic and the anaerobic oxidation of sulphydryl compounds, such as cysteine and glutathione. When this catalytic effect of the metals was prevented, either by careful purification of the sulphydryl compounds from traces of metallic impurities, or by forming a non-catalytic complex of the metals with potassium cyanide, the rate of uptake of oxygen or the rate of reduction of methylene blue by the sulphydryl compounds was found to be very much reduced. It had been claimed by Dixon and Tunnicliffe [1923] that the oxidation of sulphydryl compounds was autocatalytic. By measuring the rate of reduction of methylene blue by sulphydryl compounds, they had obtained the S-shaped curves typical of autocatalytic reactions. Further, they had shown that the aerobic and anaerobic oxidation of a sulphydryl compound was accelerated by the addition of the oxidised, *i.e.* disulphide, form, either of the same or of some other sulphydryl compound. When it was found, as indicated above, that the presence of metallic impurities ordinarily present in sulphydryl compounds exerted such a marked effect in determining the velocity of both the aerobic and anaerobic oxidation of the sulphydryl compounds, the possibility suggested itself that the accelerating effect on the aerobic and anaerobic oxidation of the SH group observed by Dixon and Tunnicliffe when a disulphide was added to the solution might in reality be due to the catalytic action of traces of metals such as iron and copper present in the added disulphide, rather than to a catalysis by the disulphide *per se*. The criticism of such a theory that it would not explain the autocatalytic curves obtained by Dixon and Tunnicliffe from measurements of the rate of reduction of methylene blue by sulphydryl compounds, does not necessarily carry much weight, since Dixon and Tunnicliffe, in a note at the end of their paper, stated that the autocatalytic nature of the curve was due partly to an initial retardation of the reaction by traces of oxygen present in the vacuum tubes. This oxygen tended to reoxidise the methylene white and led to a retardation in the initial velocity of the decoloration of the methylene blue. On taking greater precautions to remove

traces of oxygen, the curves were found to become more linear, though their autocatalytic nature never completely disappeared. It was suggested by the author [Harrison, 1924] and by Oppenheimer [1926] that traces of iron impurities might be playing a part in the catalytic effects observed by Dixon and Tunnicliffe. When, on studying the catalytic action of metals on the oxidation of the SH group, it was found that a marked acceleration was produced by concentrations of iron and copper of the order of 10^{-5} mg. per cc., or even less, it seemed worth while to attempt to find whether the catalysis observed by Dixon and Tunnicliffe was a catalysis by the disulphide itself, or was due to the catalytic action of metallic impurities present in the disulphide compound.

The following experiments suggested themselves as means of deciding this question.

(*a*) To prepare a pure disulphide compound taking every possible precaution to remove traces of metallic impurities, and to try whether this purified compound were still capable of catalysing the oxidation of sulphydryl compounds.

(*b*) To find whether the catalysis were affected by the addition of cyanides, which form non-catalytic complexes with catalytic metals.

(*c*) To destroy the —S—S— linkage by acting on the carefully purified disulphide with a suitable reagent, and then to test whether the resulting product still possessed catalytic properties. If, after this treatment, the catalysis were still produced, it would be evident that it could not be a property of the disulphide group.

Experimental.

The preparation of metal-free dithiodiglycollic acid.

Dithiodiglycollic acid was chosen as the most suitable disulphide, since it could be prepared by oxidising thioglycollic acid with iodine [Biilmann, 1905] and both the thioglycollic acid and the iodine could be obtained metal-free by distillation in quartz vessels. The iodine was purified by distillation from quartz in a current of steam. (This method was used in preference to the sublimation method of Toda [1926], since the risk of traces of metallic halides distilling over in the iodine vapour was minimised.) All the solvents used in the preparation were first distilled from quartz. 7·3 g. of thioglycollic acid (previously distilled 3 times from quartz at 16 mm. Hg pressure) was dissolved in 10 parts of pure water, and pure iodine was slowly added with shaking until a very slight excess was present. The solution was shaken with 3 volumes of ether, the ether layer being then carefully poured off and shaken with a little water. After standing, the ether layer was again poured off, the ether was evaporated and the residue dried at 100° in a quartz dish until nearly all the excess of iodine had been removed. The residue was dissolved in the minimum quantity of hot ethyl acetate, treated with 9 volumes of benzene

and allowed to crystallise. After being washed by decantation three times with a mixture of ethyl acetate and benzene, the crystals were dissolved in hot ethyl acetate and the solution poured into a quartz dish, the solvent being evaporated in a vacuum desiccator. 2·9 g. of perfectly colourless, crystalline dithiodiglycollic acid were obtained, melting at 106–107°.

The catalytic action of metal-free dithiodiglycollic acid.

Experiments were then carried out to find whether the purified dithiodiglycollic acid behaved like the unpurified disulphides in being capable of accelerating the reduction of methylene blue by sulphydryl compounds. The glass vacuum tubes employed in these experiments were well cleaned each time before use in the manner previously described [Harrison, 1927]. In all the experiments on the reduction of methylene blue, 0·3 cc. of a 1 in 5000 aqueous solution of the dye was used in each vacuum tube. The dithiodiglycollic acid was dissolved in pure water and neutralised with dilute ammonium hydroxide, previously distilled in quartz vessels. The alkali was added from a quartz burette, the amount required being determined by a separate titration. The required volume of neutralised dithiodiglycollic acid was added from the quartz burette to a mixture of 0·3 cc. of 1 in 5000 methylene blue solution, 0·1 cc. of neutralised sulphydryl compound (cysteine, thioglycollic acid or glutathione), and sufficient phosphate buffer of the required p_H to make the total volume of solution in each tube equal to 2·0 cc. In the control tubes, instead of the solution of dithiodiglycollic acid, an equal volume of pure water was added. In many of the experiments employing dithiodiglycollic acid, the latter was neutralised with sodium hydroxide instead of ammonia, since it was found that by using the former reagent the solution could be more easily maintained at the required p_H. The sodium hydroxide used was a very pure specimen made from sodium, and in every experiment the same amount of alkali, neutralised with quartz-distilled hydrochloric acid, was added to the control tube. A second control tube was used in which pure water replaced the neutralised alkali. In every case the two controls became decolorised at the same time, showing that the sodium hydroxide used to neutralise the disulphide did not contain impurities which in any way affected the rate of reaction. No special precautions were taken to obtain the sulphydryl compounds, methylene blue or phosphate buffer free from the last traces of metal impurities, since the same amount of these substances was added to both the experimental and the control tubes. The tubes were all evacuated together by means of the water-pump and filled with nitrogen (washed with alkaline pyrogallol). This was repeated and the tubes were finally evacuated completely and immersed in a water-bath maintained at the required temperature. The p_H was checked at the end of each experiment by introducing a few drops of the appropriate indicator through the side tube of the vacuum tube. The tubes in the bath were illuminated as uniformly as possible and protected from direct sunlight, since the latter appears to

accelerate the reaction. The experiments were carried out in duplicate, and the reduction times of duplicate tubes showed good agreement.

Table I gives a comparison of the time required for the reduction of methylene blue by thioglycollic acid and glutathione in the absence and in the presence of metal-free dithiodiglycollic acid (R.S.S.R.).

Table I.

R.S.S.R. = metal-free dithiodiglycollic acid. Temp. = 37°.

No.	Sulphydryl compound	p_H	Time in minutes for decoloration of methylene blue	
1	Thioglycollic acid (1·2 mg.)	7·4	Without disulphide	145
	,, ,,	,,	+4 mg. R.S.S.R.	50
2	Thioglycollic acid (1·2 mg.)	7·6	Without disulphide	110
	,, ,,	,,	+4 mg. R.S.S.R.	45
3	Thioglycollic acid (1·6 mg.)	7·6	Without disulphide	63
	,, ,,	,,	+4 mg. R.S.S.R.	23
4	*Thioglycollic acid (2·4 mg.)	7·4	Without disulphide	280
	,, ,,	,,	+4 mg. R.S.S.R.	27
	,, ,,	,,	+8 mg. R.S.S.R.	10
5	Glutathione (1·2 mg.)	7·6	Without disulphide	53
	,, ,,	,,	+4 mg. R.S.S.R.	29

* Freshly distilled thioglycollic acid.

The experimental results given in Table I are typical of a large number which have been obtained. It will be observed that a large acceleration in the rate of reduction of methylene blue by the sulphydryl compounds is produced by the addition of the purified dithiodiglycollic acid. What appears still more significant is the fact that the acceleration produced by a given amount of the metal-free disulphide is of the same order as that recorded by Dixon and Tunnicliffe using the unpurified disulphide. If the acceleration produced by the purified disulphide were due to minute traces of catalytic metals which had escaped removal, it would have been expected that the catalysis observed on adding the purified disulphide would have been of a much lower order than that produced by the unpurified, metal-containing disulphide. The fact that no appreciable reduction in catalytic power occurs, even after very careful purification, would appear to afford strong evidence that the catalysis observed is a property of the disulphide itself. The fact that the metallic impurities in the disulphide used by Dixon and Tunnicliffe caused no appreciable additional catalytic effect is probably due to the quantity of catalytic impurities already present in their unpurified —SH compounds. Still stronger evidence on these lines is given in Exp. 4 in Table I, where, using a freshly distilled specimen of thioglycollic acid with a relatively slow reducing power, the relative acceleration produced by the purified dithiodiglycollic acid was very much greater than that observed by Dixon and Tunnicliffe, using unpurified compounds. The relatively slow rate of reduction of methylene blue by the freshly distilled thioglycollic acid alone is probably due not only to removal of catalytic metals, but also to the removal, by distillation, of any traces of disulphide which might be present owing to slow oxidation of the thioglycollic acid. This would explain the relatively greater effect

produced by added disulphide. Two different preparations of dithiodiglycollic acid were made by the method given above, and both accelerated to a similar extent. This is in itself evidence that the catalysis is not due to impurities, since it is unlikely that two different preparations of the disulphide would contain the same amount of impurity.

Table II shows the effect of addition of pure dithiodiglycollic acid to sulphydryl compounds in presence of cyanide. In experiments employing potassium cyanide, a fresh solution of the latter was made up every day, and neutralised with HCl immediately before use.

Table II.

R.S.S.R. = metal-free dithiodiglycollic acid.

No.	Sulphydryl compound	p_H	Temp.	KCN	Time in minutes for decoloration of methylene blue	
1	Thioglycollic acid (1·2 mg.)	7·6	37°	*M*/500	Without disulphide	102
	" "	"	"	"	+4 mg. R.S.S.R.	62
2	*Thioglycollic acid (2·4 mg.)	7·4	37°	*M*/1000	Without disulphide	410
	" "	"	"	"	+8 mg. R.S.S.R.	17
3	*Thioglycollic acid (2·4 mg.)	7·4	37°	*M*/500	Without disulphide	420
	" "	"	"	"	+4 mg. R.S.S.R.	35
4	Cysteine (0·8 mg.)	7·6	25°	*M*/400	Without disulphide	110
	" "	"	"	"	+4 mg. R.S.S.R.	65
5	Glutathione (1·2 mg.)	7·6	25°	*M*/500	Without disulphide	173
	" "	"	"	"	+4 mg. R.S.S.R.	46

* Freshly distilled.

It is evident from Table II that the acceleration produced by pure dithiodiglycollic acid can still take place even in the presence of cyanide. Moreover, using freshly distilled thioglycollic acid as the —SH compound, the acceleration produced by the disulphide is relatively greater in the presence of cyanide than in its absence. This again affords strong evidence that the acceleration by dithiodiglycollic acid is a true property of the disulphide group itself. Potassium cyanide is well known to form non-catalytic complexes with catalytic metals such as iron and copper, and, if the observed acceleration were due to minute traces of metallic impurities which had escaped removal during purification of the disulphide, one would expect the addition of cyanide to prevent such an acceleration.

The effect of destroying the —S—S— group by bromine.

An attempt was now made to destroy the —S—S— group in the pure dithiodiglycollic acid, which, as has been shown above, is capable of catalysing the oxidation of the sulphydryl group. If, after destroying the disulphide group, the resulting product no longer catalysed the reduction of methylene blue by sulphydryl compounds, additional strong evidence would be afforded that the catalysis is a property of the disulphide group itself. Bromine was chosen as the reagent for destroying the —S—S— group in dithiodiglycollic acid by oxidation. Preliminary experiments showed that an aqueous solution of dithiodiglycollic acid readily reacts with bromine in the cold, the amount of bromine required corresponding with the formation of sulphoacetic acid.

$$\begin{matrix} S—CH_2COOH \\ | \\ S—CH_2COOH \end{matrix} \rightarrow 2 \begin{matrix} CH_2SO_3H \\ | \\ COOH \end{matrix}$$

This is analogous to the oxidation by bromine of cystine to cysteic acid.

The product no longer gave a nitroprusside reaction after treatment with zinc dust and HCl, showing that all the disulphide had been destroyed.

A dilute aqueous solution of pure dithiodiglycollic acid containing 4 mg. per cc. was treated with a slight excess of quartz-distilled bromine, the product being warmed to 110° in a quartz dish till the HBr was removed. The product was then neutralised with pure NaOH, water being added until 1 cc. of the solution contained the oxidised product from 4 mg. of dithiodiglycollic acid. The effect of 1 cc. of this solution (in which the —S—S— linkage has been destroyed by bromine) on the rate of reduction of methylene blue by thioglycollic acid was then compared with (*a*) the rate of reduction of methylene blue by thioglycollic acid alone; and with (*b*) the rate of reduction of methylene blue by thioglycollic acid in presence of 4 mg. of dithiodiglycollic acid (not treated with bromine).

The following results were obtained.

Time for reduction of methylene blue by thioglycollic acid (1·2 mg.)

$p_H = 7{\cdot}4$ Temp. = 37°

(*a*)	Alone	143 min.
(*b*)	4 mg. dithiodiglycollic acid (—S—S— linkage intact) ...	59 „
(*c*)	Product of 4 mg. of dithiodiglycollic acid treated with bromine (—S—S— linkage destroyed)	145 „

These results show that after destroying the disulphide linkage in dithiodiglycollic acid with bromine, the product has no power whatever to catalyse the anaerobic oxidation of the sulphydryl group. Had the catalysis been due to metallic impurities, these would have remained in the solution after treatment with bromine, and the catalytic power of the solution would not have been destroyed. The fact, that after destruction of the —S—S— group by bromine the catalysis is no longer manifested, clearly indicates that the catalysis is a function of the disulphide group itself.

Table III shows the effect of increasing concentrations of pure dithiodiglycollic acid on the rate of reduction of methylene blue by freshly distilled thioglycollic acid.

Table III.

Temp. = 37° $p_H = 7{\cdot}9$

Added dithiodiglycollic acid (mg.)	Time for decoloration of methylene blue by thioglycollic acid (2·4 mg.), min.
—	275
0·4	159
1·0	80
2·0	30
4·0	27

It is evident that marked acceleration is produced in the rate of anaerobic oxidation of thioglycollic acid by relatively small concentrations of pure

dithiodiglycollic acid. The fact that 4 mg. of the disulphide produces very little more acceleration than 2 mg. is probably explained by the effects of mass action, the catalytic action of the disulphide being opposed by the tendency of the latter to drive the reaction in the opposite direction. A similar effect was observed by Dixon and Tunnicliffe.

Table IV shows the catalytic effect of pure dithiodiglycollic acid at different hydrogen ion concentrations.

Table IV.

Temp. = 37°

p_H	Dithiodiglycollic acid (mg.)	Time for decoloration of methylene blue by thioglycollic acid (2·4 mg.), min.
6·0	—	255
,,	4	125
6·8	—	255
,,	4	45
7·4	—	150
,,	4	20

The results show that as the solution becomes more acid there is a falling off of the relative acceleration produced by the disulphide. Dixon and Tunnicliffe also mention this.

The catalysis produced by dithiodiglycollic acid cannot be attributed to an appreciable increase in the concentration in the —SH group, brought about by hydrolysis at the —S—S— linkage in the weakly alkaline solutions, since it was found that dithiodiglycollic acid alone showed no ability to reduce methylene blue even on prolonged standing in the bath at 37°. Further, in some of the experiments, using tubes with and without added disulphide, the tubes were opened, 0·1 cc. of concentrated HCl was quickly added to each (to prevent further oxidation), and the solutions were titrated with $N/100$ iodine, using starch as an indicator. The tubes containing added disulphide were found to reduce practically the same amount of iodine as those containing no added disulphide. This shows that any hydrolysis of the disulphide which occurs is of so slight an extent that the amount of —SH formed, in comparison with that already present, is too small to be detected by the above experiment. Even in the presence of KCN, which is known to increase the hydrolysis of the —S—S— group, no decoloration of methylene blue was observed. (5·2 mg. dithiodiglycollic acid, $M/200$ KCN, methylene blue and phosphate buffer of p_H 8·0.)

Ample justification for undertaking the work described in this paper is afforded by the fact that ordinary specimens of so-called pure substances chosen at random, differing as widely as succinic acid and ammonium chloride, were found to be capable of causing an acceleration in the anaerobic oxidation of thioglycollic acid of a similar order to that produced by an equal weight of dithiodiglycollic acid. After subliming the succinic acid as succinic anhydride into a quartz dish and neutralising with pure sodium hydroxide, it was found to be no longer capable of producing any acceleration in the reaction.

The acceleration produced by ammonium chloride did not disappear after sublimation into quartz vessels, but a specimen prepared by neutralising quartz-distilled hydrochloric acid with quartz-distilled ammonia did not produce any acceleration in the reduction of methylene blue by thioglycollic acid. (Probably the failure to destroy the catalytic power of ammonium chloride by sublimation was due to the simultaneous sublimation of the halides of catalytic metals in the atmosphere of dry HCl produced by the dissociation of the ammonium chloride.) It is not surprising that so-called pure substances should be capable of producing such an acceleration in the oxidation of sulphydryl compounds, when one considers the powerful catalytic effect produced on the reaction by extraordinarily minute traces of such metals as copper and iron.

The catalytic action of metal-free dithiodiglycollic acid on the aerobic oxidation of sulphydryl compounds.

Experiments were carried out to find whether the metal-free dithiodiglycollic acid was capable of catalysing the oxidation by oxygen of thioglycollic acid and cysteine. The oxygen uptake was measured by means of the Barcroft microrespirometer, the apparatus being shaken in a water-bath at 37°. The bottles of the apparatus were made of quartz, and were thoroughly cleaned with acids before use. The total volume of liquid in each bottle was 3·0 cc., this volume being made up with phosphate buffer. The dithiodiglycollic acid was neutralised with pure NaOH, an equal amount of the alkali, neutralised with quartz-distilled HCl, being used in the control apparatus.

Fig. 1 shows the catalytic effect produced on the aerobic oxidation of cysteine by the addition of pure dithiodiglycollic acid. It is evident that a large acceleration is produced by the addition of the pure disulphide, and, further, by comparing curves *B* and *C*, it can be seen that an acceleration is brought about by the disulphide even in presence of *M*/900 cyanide. It is very significant that curves *A* and *B* show distinctly the **S**-shaped, sinuous form characteristic of an autocatalytic reaction. Dixon and Tunnicliffe failed to obtain any such evidence of the autocatalytic nature of the reaction in any of their experiments on the aerobic oxidation of sulphydryl compounds. In the case of cysteine, they explain this by the fact that the disulphide formed, namely, cystine, is insoluble, and, being precipitated from the solution, is unable to catalyse the oxidation of the remaining cysteine. In the present experiment, such a precipitation of cystine was observed during the oxidation of the control, containing no added disulphide (Curve *C*), but it was interesting to find that in the two solutions containing added disulphide (Curves *A* and *B*), no precipitation of cystine could be observed, even at the end of the reaction. This ability of dithiodiglycollic acid to prevent the precipitation of cystine is probably due to a mutual interaction of the two disulphides, cystine and dithiodiglycollic acid, of the nature:

$$\text{R.S.S.R.} + \text{R}'.\text{S.S.R}'. \rightarrow 2\ \text{R.S.S.R}'.,$$

leading to the formation of a soluble product. Hence, during the reaction, the concentration of soluble disulphide is continuously increasing, and one observes not only the catalytic effect of added dithiodiglycollic acid, but also the autocatalytic effect of the increasing concentration of cystine formed during the reaction and held in solution by the dithiodiglycollic acid. The fact that such an autocatalytic curve has now been obtained for the *aerobic* oxidation of a sulphydryl compound affords almost conclusive evidence that the oxidation of the sulphydryl group is catalysed by disulphides.

The control curve *C* appears to be very slightly autocatalytic in shape. This may be due to non-precipitation of cystine in the earlier stages of the reaction, owing to slight solubility or to supersaturation of the solution (such a retention in solution of small quantities of cystine has been observed in the methylene blue experiments, in which no precipitation of cystine could be observed at the end of the reaction); or it might possibly be due to removal

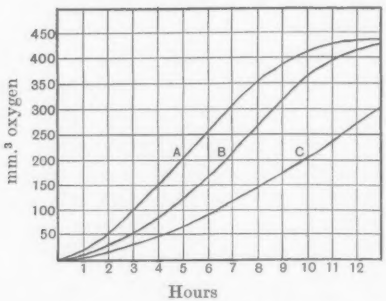

Fig. 1. A. 8 mg. cysteine + 16 mg. pure dithiodiglycollic acid
B. „ „ + „ „ „ „ + *M*/900 KCN
C. „ „ + *M*/900 KCN
$p_H = 7{\cdot}4$.

of a small amount of cyanide by distillation into the upper part of the bottle, or even by coupled oxidation or decomposition during the reaction. This might allow a slight increase in the activity of traces of catalytic metals present in the cysteine. A similar effect might account for a very small part of the autocatalysis exhibited by curve *B*, but the autocatalytic nature of curve *A* must be due entirely to a catalysis by disulphide since cyanide was not present.

It may be mentioned that in an unpublished experiment carried out by the author in 1924 it was found that the addition of 45 mg. of oxidised glutathione (freed as completely as possible from catalytic metals by the method described [Harrison, 1924]) to 13·5 mg. of pure cysteine hydrochloride, brought about a marked acceleration in the rate of oxidation of the latter (temp. = 37°; $p_H = 7{\cdot}6$; vol. = 3 cc.). Here again it was observed that no precipitation of cystine occurred in the bottles containing added oxidised

glutathione, though in this case, owing to the rapid oxygen uptake, the readings taken were not sufficiently accurate to determine whether or not the reaction was autocatalytic.

It has been found that the aerobic oxidation of thioglycollic acid is also catalysed by the addition of pure dithiodiglycollic acid. 8 mg. of thioglycollic acid in 3 cc. of water (temp. = 37°, $p_H = 7{\cdot}4$), took up 13·7 mm.^{3} of oxygen in the first 11 hours, while an equal amount of thioglycollic acid to which 16 mg. of pure dithiodiglycollic acid had been added took up 72·4 mm.^{3} of oxygen over the same period of time.

Discussion.

It has been shown by the experiments described that the catalytic effect produced in both the aerobic and anaerobic oxidation of sulphydryl compounds by the addition of disulphides is not due to catalytic impurities present in the disulphide, but is a true property of the disulphide itself. The question arises as to explanation of this catalytic effect. Dixon and Tunnicliffe advance the hypothesis that an addition compound between the sulphydryl compound and the disulphide is formed, and that the compound oxidises more rapidly than the sulphydryl compound itself. The evidence they give in support of this hypothesis is very slender, and definite reasons can be given against the likelihood of the formation of such an addition compound. Sidgwick [1927] has recently pointed out that certain substances, such as water and the alcohols, show many abnormal physical properties, owing to the formation of complexes by molecular association. When the oxygen in these compounds is replaced by sulphur, the resulting compounds show normal physical properties, indicating that association has not taken place. Unlike oxygen compounds, sulphur compounds do not show a tendency to complex formation.

It seems possible that the catalytic effect of the disulphide compounds on the oxidation of the sulphydryl compounds may be explained by the following mechanism. Firstly, the disulphide, when in solution, is, to a very slight extent hydrolysed to a mixture of the corresponding sulphydryl compound and the sulphenic acid:

$$(1)\quad \text{R.S.S.R.} + \text{H}_2\text{O} \rightleftarrows \text{R.SH} + \text{R.SOH}.$$

Secondly, the sulphenic acid takes up oxygen to form the sulphinic acid:

$$(2)\quad \text{R.SOH} + \text{O} \rightleftarrows \text{R.SOOH}.$$

Thirdly, if a sulphydryl compound (R′SH) is present, it reduces the sulphinic acid:

$$(3)\quad 2\text{R′SH} + \text{R.SOOH} \rightleftarrows \text{R′.S.S.R′.} + \text{R.SOH}.$$

The sulphydryl compound thus becomes oxidised to the disulphide, and the sulphenic acid is regenerated. If the oxidation of the sulphenic acid to the sulphinic acid, and the re-reduction of the latter by the sulphydryl compound, together take place more rapidly than the direct oxidation of the sulphydryl

compound by the air (and experiments have shown that the latter reaction in the absence of metals is very slow), then a positive catalysis of the oxidation will be expected on the addition of disulphide.

That such reactions as those suggested above can actually take place appears likely from the following considerations. Reaction (1), namely, the hydrolysis of the —S—S— group to a mixture of —SH and —SOH is given by Smiles [1911] as the way in which the breakdown of aromatic disulphides in acid or alkaline solution takes place. Evidence is given by Smiles and Stewart [1921] that in the case of the hydrolysis of *m*-dithiobenzoic acid (which takes place on treatment with cold aqueous NaOH) the primary products are the sulphydryl compound and the sulphenic acid.

The isolation of the sulphenic acids has not apparently been achieved so far, except in the case of anthraquinonesulphenic acid [Fries, 1912], but derivatives can be isolated. Smiles points out that the hydrolysis (1) is reversible and that the equilibrium is greatly in favour of the disulphide. As regards reaction (2), it has been shown that anthraquinonesulphenic acid in solution readily takes up oxygen from the air to form the corresponding sulphinic acid. (Indeed the occurrence of sulphinic acid in alkaline solutions of a disulphide [Fromm and Wittmann, 1908] was one of the reasons for suggesting the existence of sulphenic acids.) There is little doubt that reaction (3), namely, the reduction of the sulphinic acid by the sulphydryl compound can take place, for Otto [1888] has shown that phenylsulphinic acid is reduced by hydrogen sulphide, and Gilman, Smith and Parker [1925] find that even toluenesulphonyl chloride is reduced by R.SH. Many of the aliphatic sulphinic acids have been isolated, and the fact that the existence of the aliphatic sulphenic acids is probably only transitory does not in any way mean that they are necessarily incapable of taking part in such a catalysis as that pictured above.

In the case of the anaerobic oxidation of sulphydryl compounds in which methylene blue replaces oxygen, the catalysis by disulphides may be assumed to take place on exactly similar lines, the reversible reaction

$$(2\,a)\quad \mathrm{R.SOH} + \mathrm{Mb} + \mathrm{H_2O} \rightleftarrows \mathrm{R.SOOH} + \mathrm{Mb.H_2}$$

being substituted for reaction (2). Reaction (2 *a*) will proceed in a forward direction leading to complete reduction of the methylene blue, provided that the sulphinic acid R.SOOH is continuously removed by re-reduction by the sulphydryl compound.

It is not essential that the actual compounds concerned in the intermediate reactions should necessarily be those mentioned. It is merely suggested that the formation of compounds of such a type may be responsible for the catalysis by disulphides.

If the catalysis by disulphides takes place by the mechanism described, in which the first stage involves hydrolysis of the disulphide, it might be expected, since this hydrolysis takes place to a greater extent in alkaline solution, that increasing the p_{H} of the solution would bring about an increase

in the catalytic power of the disulphide. That such is actually the case is evident from the experiment shown in Table IV. This increase of catalysis in alkaline solution thus affords some evidence for the theory suggested.

Previously quoted experiments indicate that near neutrality, if hydrolysis of the —S—S— group occurs, it does so to a very small extent. In other words, reactions (1) and (2) proceed in a forward direction only very slightly. This does not in any way preclude the possibility that hydrolysis may occur to a sufficient extent to bring about the catalysis.

Considering the experiments as a whole, there seems no doubt that the catalytic effect of disulphides on the oxidation of sulphydryl compounds is a true property of the —S—S— group and is not due to metal impurities.

SUMMARY.

(1) Both the aerobic and anaerobic oxidations of sulphydryl compounds are catalysed by pure (metal-free) dithiodiglycollic acid.

(2) This catalysis is not inhibited by cyanide.

(3) By destroying the —S—S— linkage in pure dithiodiglycollic acid, its catalytic action disappears.

(4) A mechanism is suggested by which the catalysis may take place.

The author wishes to thank Prof. E. Mellanby for his interest and encouragement during the course of this work and Dr G. M. Bennett for helpful discussion on the sulphur compounds.

He also wishes to express his indebtedness for a Keddey Fletcher-Warr Studentship, University of London, held during the course of this work.

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CLXXXV. THE USE OF THE STARCH-IODINE END-POINT IN TUNNICLIFFE'S METHOD FOR THE DETERMINATION OF GLUTATHIONE IN TISSUES.

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(*Received November 7th, 1927.*)

In the first publication of his admirably simple method for the estimation of glutathione in biological material Tunnicliffe [1925] observed that if it were attempted to carry out the iodine titration of the trichloroacetic acid filtrate with starch as an internal indicator, figures were obtained which were substantially higher than those yielded with nitroprusside used as an external indicator. This finding was later corroborated by Voegtlin and Thompson [1926] and more recently by Kennaway and Hieger [1927]. On the other hand, Uyei [1926] claims to have obtained correct figures with starch as an internal indicator, giving analyses for labile sulphur in support of his claim.

In considering the possible causes for this discrepancy between the nitroprusside and starch titration figures it occurred to the writers that one of the indispensable conditions for the achievement of a good starch-iodine end-point was lacking in the procedure as given by Tunnicliffe. It will be recalled that the titration is carried out with very dilute, $N/100$, or even more dilute, iodine solutions. Such solutions are usually prepared by dilution from $N/10$ stock iodine solutions which contain a certain amount, usually 25 g. per litre, of potassium iodide. Therefore, in the titration of the glutathione with $N/100$, or with more dilute, iodine solutions practically no potassium iodide is present. That the presence of a considerable excess of iodide in acid solution is necessary for the production of a relatively permanent blue starch-iodine compound is an old and well-established fact in analytical chemistry.

In applying this principle to the determination of glutathione by Tunnicliffe's method in various tissue extracts it was found that a strikingly close correspondence between the nitroprusside and starch figures could be obtained, as demonstrated in Table I.

The modification simply consists in adding to the trichloroacetic acid extract to be titrated about 2 cc. of a 25 % solution of potassium iodide

(0·5 g.). Since the disappearance of the blue colour from a solution containing an excess of iodine on the addition of thiosulphate appears to be an even more sensitive reaction than the appearance of the blue colour with iodine, it was found advisable to add a known excess of the iodine solution and to titrate back with an equivalent thiosulphate solution.

The advantages of using the extremely delicate starch-iodine end-point, and of an internal rather than an external indicator, are obvious and require no further comment, beyond drawing attention to our observation, as shown in Table I, that much smaller quantities of glutathione can be determined by means of titration with 0·001 *N* iodine and thiosulphate solutions than could possibly be accomplished with the nitroprusside end-point, which is extremely unsatisfactory for small concentrations of glutathione.

Table I.

Trichloroacetic acid extract		Nitroprusside end-point		Starch end-point without KI		Modified titration with $Na_2S_2O_3$ and added KI, starch end-point	
Tissue	Amount used cc.	cc.	I solution normality	cc.	I solution normality	cc.	I solution normality
Rabbit liver	5·0	2·10	0·005	2·37	0·005	2·05	0·005
Rabbit liver	5·0	3·09	0·005	3·32	0·005	3·04	0·005
	5·0	3·08	„	3·37	„	3·07	„
Rabbit liver	10·0	5·12	0·005	5·80	0·005	5·21	0·005
Mackerel liver	6·0	1·19	0·004	1·31	0·004	1·16	0·004
	3·0	—	—	—	—	0·58	„
Mackerel liver	5·0	0·92	0·005	1·28	0·005	0·96	0·005
	5·0	0·90	„	1·23	„	0·91	„
	5·0	0·90	„	1·25	„	0·91	„
	10·0	(1·82 calc'd	„)	2·21	„	1·79	„
Mackerel liver	10·0	0·27	0·004	0·59	0·004	0·27	0·004
	5·0	(0·54 calc'd	0·001)	1·11	0·001	0·56	0·001
	10·0	(1·08 „	„)	1·83	„	1·15	„
Dogfish liver	15·0	1·47	0·005	1·85	0·005	1·54	0·005
	15·0	1·50	„	1·87	„	1·50	„
Human red blood cells	10·0	1·21	0·002	1·48	0·002	1·20	0·002
Yeast	5·0	3·40	0·002	4·10	0·002	3·45	0·002
	5·0	3·44	„	4·16	„	3·42	„

Although the proposed modification offers a more convenient and perhaps more accurate analytical procedure, the possibility of the presence in tissue extracts of substances other than glutathione which are capable of combining with iodine must not be overlooked. It is certainly advisable in any series of determinations to make a preliminary comparison of the KI-starch-iodine titration with the nitroprusside-iodine method, employing preferably quite concentrated glutathione solutions, to make certain that no such substances are encountered. In a rather extensive series of determinations in trichloroacetic acid extracts of animal tissues the present writers have not encountered any such discrepancies, with the single exception of bile-containing liver extracts, in which case the starch-iodine method yielded somewhat higher figures.

SUMMARY.

The addition of potassium iodide to trichloroacetic acid extracts of tissues permits the employment of the delicate starch-iodine end-point in the titration of glutathione by Tunnicliffe's method.

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CLXXXVI. A NOTE ON THE HYPOGLYCAEMIC ACTION OF DIHYDROXYACETONE IN MAN.

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(*Received November 7th, 1927.*)

Several interesting and apparently conflicting observations have accumulated about dihydroxyacetone.

(I) When administered to dogs [Campbell and Soskin; Himwich, Rose and Malev, 1926] or human beings [Cathcart and Markowitz, 1927], it causes a much prompter rise in the R.Q. to unity or over than does an equivalent quantity of glucose.

(II) It augments considerably the hyperglycaemia of depancreatised dogs, by whom it is quantitatively excreted as glucose. During this process the R.Q. shows a slight fall [Campbell and Markowitz, 1927].

(III) It exerts no curative influence on the hypoglycaemia following hepatectomy [Markowitz and Campbell, 1927].

(IV) When injected into eviscerated nephrectomised preparations, it enters unchanged into the tissues, the concentration of this substance in the tissues, at the end of several hours (as calculated from the concentration of the triose in a muscle), being sufficient to account for most of the triose administered [Markowitz and Campbell, 1927].

(V) It has no influence on the respiratory metabolism of excised amphibian muscle [Meyerhof, Lohman and Meier, 1925].

Observation (I) suggests that the triose is burnt with much greater facility than glucose. However, observations (II)–(V), if taken in conjunction, make it reasonably certain that dihydroxyacetone cannot be used as such, but must first be converted into glucose by the liver. The explanation for the high R.Q. following the administration of the triose is somewhat conjectural, but (as stated in our previous communication [Cathcart and Markowitz, 1927]) we believe it to depend on the following consideration. A depancreatised dog of only about 6 kg. can excrete nearly 20 g. of dihydroxyacetone as glucose in less than 6 hours. It is therefore a fair assumption that, following the administration of the triose to the intact animal, the tension of carbohydrate in the liver is considerably increased. Presumably the increase is sufficient not only to stop glyco-neogenesis out of fat and protein, but also to bring about a considerable synthesis of fat.

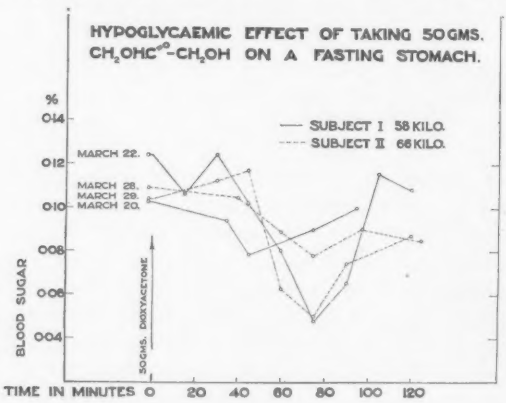
In agreement with the finding that dihydroxyacetone is a sugar-former in the animal body is the observation that it can act as an antidote to the hypoglycaemia following overdosage with insulin both in man and the lower animals [Campbell and Hepburn, 1926; Kermack, Lambie and Slater, 1926]. In decided contrast to this latter property of dihydroxyacetone is the observation, first made by Isaac and Adler [1924] and confirmed by Rabinowitch [1925], that the triose has a hypoglycaemic action in the human subject.

In the course of an investigation which had quite a different object, we found that, following the ingestion of 50 g. dihydroxyacetone on a *fasting* stomach, the subject developed muscular tremor, flushing, restlessness and general malaise. The condition was not recognised until routine blood-sugar determinations, which were made during the course of the experiments, revealed that the symptoms coincided with the existence of hypoglycaemia.

Since dihydroxyacetone thus resembles glucose in that it can both relieve insulin hypoglycaemia and definitely induce hypoglycaemia in man, it was deemed necessary to confirm its blood-sugar-lowering properties.

Methods.

Five experiments in all were done on two subjects. One of these experiments was reported in Fig. 5 of our previous communication [1927]. The results of the remaining four experiments are presented in the form of a chart. Blood-sugars were estimated by MacLean's method. No correction for the presence of dihydroxyacetone in the first few samples has been introduced. Mason [1926], Campbell (personal communication) and others have shown that the quantity appearing in the blood after such doses is insignificant.



Discussion.

It requires to be explained why a substance which forms sugar so readily in the animal body should have a hypoglycaemic action. The possible mechanism of this action can as yet be only conjectured.

The sugar of the blood is normally a balance between (1) the production of sugar by the liver [Soskin, 1927], (2) the utilisation or storage of sugar by the tissues, and (3) the secretion of insulin by the pancreas. A fall in the blood-sugar must be due to one or more of these factors, each of which will be discussed in turn.

(1) Although there is a considerable rise in the oxygen consumption immediately following the administration of dihydroxyacetone, this is by no means comparable to that occurring even in mild muscular exercise, in which condition there is no hypoglycaemia. It is, therefore, justifiable to conclude that the hypoglycaemia is not due to the increased energy requirement of the organism, although this latter may be a contributory cause.

(2) Before one can state that the hypoglycaemia of dihydroxyacetone is not insular in origin, it must be shown that when administered to *surgically* hepatectomised dogs, it does not accelerate the rate of fall of the blood-sugar, which it should do if it evoked a secretion of insulin in normal animals. Markowitz and Campbell [1927] found that when injected into a *functionally* hepatectomised dog, there was no evidence that the triose accelerated the rate of fall of the blood-sugar. However, this effect could have been masked by the presence of a slight amount of functioning liver tissue.

In favour of the insular origin of the hypoglycaemia following dihydroxyacetone may be mentioned the observation that the administration of the triose considerably augments the hyperglycaemia of a depancreatised dog. This finding, however, may possibly be dependent on the inability of the diabetic liver to hold glycogen, and not on the absence of insulin *per se*.

The ability of dihydroxyacetone to induce hypoglycaemia may be compared with the similar ability of glucose, under certain conditions, to bring about hypoglycaemia [Gibson and Larimer, 1924]. The administration of 50 g. glucose to a fasting normal human subject is regularly followed by hypoglycaemia after the initial hyperglycaemia has passed away. In some cases the hypoglycaemia is so profound as to cause symptoms like those of insulin overdosage. There is some evidence that the hypoglycaemia from glucose is dependent on an over-secretion of insulin. It is possible that dihydroxyacetone brings about hypoglycaemia in a similar fashion, although there is no evidence for this as yet.

(3) The liver is quite possibly concerned with the hypoglycaemia of dihydroxyacetone. Since the mechanism of the normal constant secretion of glucose into the blood by the liver is not known, the possible mechanism of this can only be vaguely conjectured. In our previous communication we put forward the tentative hypothesis that the extraordinarily high R.Q. following the administration of dihydroxyacetone was dependent on an increased tension of carbohydrate in the liver, an increase so great as not only to stop glyco-neogenesis out of fat and protein, but also to result in a considerable synthesis of fat. In the light of present knowledge it seems possible that a similar explanation may obtain in the case of the hypoglycaemic action

of this triose. Normally the cause for a greatly increased tension of carbohydrate in the liver is an excessive quantity of sugar of alimentary origin in the blood. Under these conditions it is possible that the hepatic secretion of blood-sugar ceases. When the tension of carbohydrate in the liver increases from any other cause, it is possible that an inhibition of the blood-sugar-secreting function of the liver ensues, which soon leads to hypoglycaemia, since the existing blood-sugar becomes rapidly used up.

In any case, there is no evidence for regarding the hypoglycaemic action of dihydroxyacetone in man as other than an adventitious one, being in this regard similar to glucose. It is therefore not dependent on any anti-diabetic qualities that the substance may possess. This viewpoint is in harmony with the fact that neither anti-diabetic nor hypoglycaemic functions of the triose have been demonstrable in the lower animals.

We should like to express our indebtedness to Miss E. L. Weatherhead for help with the blood-sugar determinations in these experiments.

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CLXXXVII. THE DIURNAL VARIATION OF THE GASEOUS CONSTITUENTS OF RIVER WATERS. PART II.

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(*Received November 7th, 1927.*)

The work described in Part I [1927] in connection with the diurnal variation of the gaseous constituents of river waters has been continued throughout the summer of 1927, and the following experiments over periods of 24 hours have now been performed.

River Lark.	Series	I. March 30th–31st
	,,	II. April 27th–28th
	,,	III. May 18th–19th
	,,	IV. June 28th–29th
	,,	IV *a*. June 30th
	,,	V. August 4th–5th
River Itchen.	Series	I. April 6th–7th
	,,	II. May 4th–5th
	,,	III. May 31st–June 1st
	,,	IV. July 5th–6th
	,,	V. July 27th–28th

The results obtained in March and April on the River Lark and in April and May on the River Itchen have been given and discussed in Part I, while the remainder are here tabulated as follows:

Table	VI.	River Lark.	May 18th–19th.	Fig.	5
,,	VII.	,,	June 28th–29th.	,,	6
,,	VII (*a*).		June 30th.		
,,	VIII.	,,	August 4th–5th.	,,	7
,,	IX.	River Itchen.	May 31st–June 1st.	,,	8
,,	X.	,,	July 5th–6th.	,,	9
,,	XI.	,,	July 27th–28th.	,,	10

The times and values of the maxima and minima of the gaseous constituents on the River Lark are tabulated in Table XII, together with a summary of the weather conditions and river vegetation on the dates of the experiments.

Table VI. *River Lark. May 18th–19th, 1927. Series III.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
6 p.m.	15·0	1·696	165	0·01–0·015	8·0	Bright sun. Floating diatoms
7 „	14·5	1·567	151	0·01–0·015	8·0	Dull. Floating diatoms
8 „	14·25	1·451	139	0·01–0·015	8·0	Light failing
9 „	14·0	1·255	119·5	0·01–0·015	7·9–8·0	Dusk
10 „	13·4	1·099	104	0·01–0·015	7·8	Fine. Dark. Clear
11 „	13·2	0·977	92	0·015	7·7	Full moon rising. Fine
Midnight	13·0	0·824	77	0·015	7·6	Moonlight. Fine
1 a.m.	12·6	0·744	69	0·02	7·5–7·6	„ „
2 „	12·1	0·675	61	0·02–0·03	7·5–7·6	„ „
3 „	11·75	0·631	57	0·02–0·03	7·5	Sky lightening. Cloudy
4 „	11·6	0·519	47	0·08	7·5	Dull. Misty
5 „	11·0	0·419	37	0·16	7·5	Cloudy. Misty
6 „	11·0	0·407	36	0·16–0·18	7·5	Cloudy
7 „	10·8	0·431	38	0·16–0·18	7·5	Dull
8 „	10·8	0·448	40	0·16–0·18	7·6	Sun breaking through clouds
9 „	11·25	0·550	49	0·16–0·18	7·6	Bright sun
10 „	12·0	0·681	62	0·2	7·6	„
11 „	12·0	0·894	81	0·18–0·2	7·6–7·7	„
Midday	13·8	1·053	100	0·18–0·2	7·7	„
1 p.m.	14·0	1·345	128	0·16	7·8–7·9	„
2 „	14·6	1·569	151	0·08	7·8–7·9	„
3 „	14·6	1·734	167	0·04	7·9–8·0	„
4 „	15·0	1·851	180	0·03	8·0	„
5 „	15·4	1·844	181	0·02	8·0	Cooler. Bright
6 „	15·2	1·822	177	0·01–0·015	8·0	„ „

Sunset May 18th, 7.48 p.m.
Sunrise „ 19th, 4.4 a.m.

Table VII. *River Lark. June 28th–29th, 1927. Series IV.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
10 a.m.	12·6	0·618	57	0·16	7·5–7·6	Dull. Cloudy. Drizzle
11 „	12·75	0·682	63	0·12–0·13	7·6	„ „ „
Midday	13·2	0·828	77	0·07–0·08	7·6	„ „ „
1 p.m.	13·4	0·906	85	0·04	7·7	„ „ „
2 „	14·2	1·033	98	0·025–0·03	7·7–7·8	Sun through
3 „	14·5	1·140	110	0·015	7·8	„
4 „	14·3	1·153	111	0·015	7·8–7·9	Heavy rain. Dull
5 „	14·25	1·103	106	0·015	7·9	Drizzle. Rainstorm
6 „	14·2	1·063	102	0·01–0·015	7·9	Dull. Cloudy
7 „	14·1	1·073	102	0·01–0·015	7·9–8·0	„ „
8 „	13·8	1·033	98	0·01	7·9	Sunset
9 „	13·7	0·963	91	0·01–0·015	7·9	Dusk
10 „	13·5	0·886	84	0·01–0·015	7·8	Almost dark
11 „	13·5	0·824	78	0·01–0·015	7·7	Dark. Raining
Midnight	13·4	0·786	74	0·01–0·015	7·6–7·7	Clearer
1 a.m.	13·4	0·744	70	0·015	7·6	Fine. Cloudy
2 „	13·1	0·688	64	0·015–0·02	7·5–7·6	Sky just lightening
3 „	13·0	0·604	56	0·04	7·5	Light. Cloudy
4 „	12·8	0·489	45	0·08	7·5	Cloudy
5 „	12·8	0·427	40	0·13	7·4–7·5	Sun up
6 „	12·75	0·399	37	0·16	7·4–7·5	Sun through. Fine. Eclipse
7 „	12·75	0·420	39	0·16	7·4–7·5	Rain. Dull
8 „	12·7	0·482	45	0·16–0·18	7·4–7·5	„ „
9 „	12·7	0·514	48	0·18	7·5	Drizzle. Dull
10 „	13·1	0·590	55	0·16	7·5–7·6	Dull. Fine

Sunset June 28th, 8.21 p.m.
Sunrise „ 29th, 3.45 a.m.
Sunset „ 29th, 8.21 p.m.

Table VII (*a*). *River Lark. June 30th*, 1927. *Series IVa.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation of dissolved oxygen	Remarks
2 p.m.	15·3	0·93	91	
3 ,,	15·4	1·004	98	
4 ,,	15·7	1·063	105	Afternoon considerably more sunny than that of June 29th
5 ,,	15·9	1·114	110	
6 ,,	16·2	1·093	108	
7 ,,	15·9	1·091	108	
8 ,,	15·6	1·051	103	

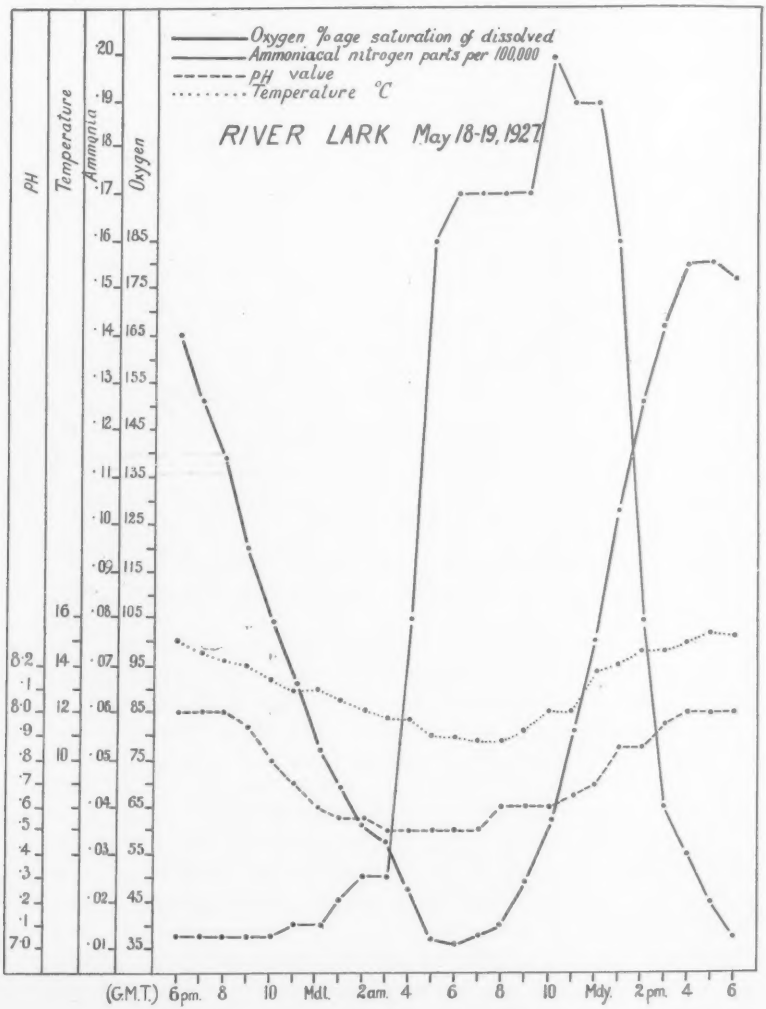

Fig. 5.

Table VIII. *River Lark. August 4th–5th*, 1927. *Series V.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
9 a.m.	16·25	0·579	58	0·01	7·5–7·6	Bright sun after bright morning
10 „	16·3	0·654	65	0·01	7·6–7·7	Bright sun
11 „	16·65	0·801	80	0·01–0·015	7·8	„
Midday	17·6	0·971	99	0·015–0·02	7·9	„
1 p.m.	17·9	1·079	111	0·015–0·02	8·0	Cloudy. Occasional sun
2 „	18·0	1·171	121	0·015–0·02	8·0–8·2	Cloudy. Water level falling rapidly. Mill stopped just before 2 p.m. and level rose again
3 „	18·1	1·148	118	0·015–0·02	8·0–8·2	Cloudy
4 „	18·2	1·174	121	0·015–0·02	8·0–8·2	Less cloud
5 „	18·4	1·197	124	0·015	8·0–8·2	Clear. Sunny
6 „	18·2	1·162	120	0·01–0·015	8·0–8·2	Sunny
7 „	17·85	1·060	109	0·01	8·0–8·2	Sun setting
8 „	17·6	1·038	106	0·01	8·0	Dusk. Fine
9 „	17·1	0·990	100	0·01	8·0	
10 „	16·9	0·891	90	0·01	7·8–7·9	
11 „	16·8	0·819	83	0·01	7·8	Clear
Midnight	16·8	0·772	77	0·01	7·7–7·8	„
1 a.m.	16·35	0·708	71	0·01	7·7–7·8	Fine
2 „	16·2	0·678	67	0·01	7·6–7·7	Sky just lightening
3 „	16·1	0·603	60	0·01	7·6	Dawn
4 „	15·9	0·534	53	0·01	7·5–7·6	Daylight. Fine
5 „	15·8	0·493	49	0·01	7·5	Misty
6 „	15·8	0·458	45	0·01	7·5	„
7 „	15·7	0·428	42	0·01	7·5	„
8 „	15·8	0·442	44	0·01	7·5	Dull
9 „	16·1	0·528	53	0·01	7·5–7·6	Sunshine

Sunset August 4th, 7.45 p.m.
Sunrise „ 5th, 4.28 a.m.
Sunset „ 5th, 7.43 p.m.

Oxygen curves.

The oxygen curves of May, June and August are in general similar to those obtained in March and April, although the times of the maximum and minimum values vary to a certain extent.

One noticeable feature of the oxygen curves (R. Lark) is the progressive shortening of the period of minimum of oxygen from March (6 hours) and April (3 hours) to May (2 hours), while July and August give a single minimum value. These differences may be accounted for by the variation of the duration of the periods of darkness at the different dates.

Confirmatory results were not obtained on the Itchen since consistent small irregularities of samples were caused by the turbulence of this stream.

The time of the increase from the minimum value does not appear to be influenced by the time of sunrise so much as by the meteorological conditions.

It would appear that the current weather conditions have a considerable effect upon the times of the maximum and minimum values, and the results of an experiment performed during Series IV *a* on the River Lark bear out this point: on June 29th, after a dull day with intermittent rain, the maximum

oxygen values (111 %, 110 %) were obtained at 3 and 4 p.m., whereas on the following day, June 30th, after an afternoon considerably more sunny than that of the previous day, the maximum values (110 %, 108 %, 108 %) were obtained from 5–7 p.m.

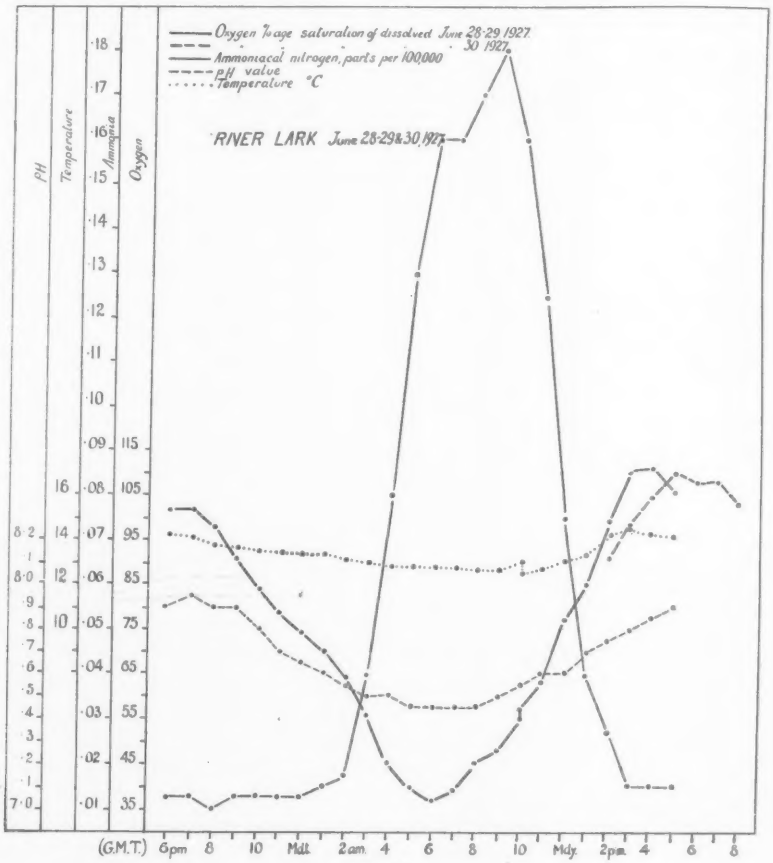
Oxygen % age saturation of dissolved June 28-29 1927
30 1927
Ammoniacal nitrogen, parts per 100,000
pH value
Temperature °C
RIVER LARK June 28-29 & 30, 1927
pH
Temperature
Ammonia
Oxygen
(G.M.T.) 6pm 8 10 Mdt. 2am 4 6 8 10 Mdy. 2pm 4 6 8

Fig. 6.

Considerable differences were noted in the maximum values of dissolved oxygen obtained on the River Lark; the March, April and May maxima were respectively 157, 178, and 181 % of saturation, and it is difficult at this stage to assign these differences definitely to the effects of either different actinic conditions or different conditions of plant life. The June and August maxima of 111 and 124 % respectively are considerably lower than those of March, April and May, and it is safe in conjunction with the botanical data (see p. 1433) to account for the difference by the decrease in the number of diatoms present.

Table IX. *River Itchen. May 31st–June 1st, 1927. Series III.*

Time G.M.T.	Temp. °C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
10 a.m.	10·6	1·145	101	0·005	7·8	Dull showery. Water turbid
11 „	11·3	1·206	109	0·005	7·8	„ „
Midday	12·3	1·257	116	0·005	7·8	„ „
1 p.m.	12·5	1·172	109	0·005	7·8	„ „
2 „	12·8	1·245	116	0·005	7·9	„ „
3 „	13·5	1·192	112	0·005	7·85	„ „
4 „	13·9	1·135	108	0·005	7·85	Sunshine. „
5 „	14·3	1·201	115	0·005	7·85	„ „
6 „	14·0	1·115	106	0·005	7·85	„ „
7 „	13·9	1·103	105	0·005	7·8	„ „
8 „	13·0	1·004	94	0·005	7·8	Sunset. „
9 „	12·7	0·895	83	0·005	7·8	Cloudy. Almost dark
10 „	12·5	0·871	81	0·005	7·7	„ Dark
11 „	12·1	0·886	81	0·005	7·7	„ „
Midnight	12·0	0·827	76	0·005	7·7	„ Slight drizzle
1 a.m.	12·0	0·859	79	0·005	7·7	„ „
2 „	11·7	0·829	75	0·005	7·7	Cloudy. Slight drizzle. Sky lightening
3 „	11·5	0·852	77	0·005	7·6	Dawn. Slight drizzle
4 „	11·3	0·833	75	0·005	7·6	Dull. „
5 „	11·1	0·846	76	0·005	7·6	„ „
6 „	11·0	0·935	86	0·005	7·6	„ Fine
7 „	11·0	0·913	83	0·005	7·6	„ „
8 „	11·0	1·060	94	0·005	7·75	„ „
9 „	11·5	1·168	105	0·005	7·8	„ „
10 „	11·9	1·177	108	0·005	7·9	„ „

Sunrise May 31st, 3.50 a.m.
Sunset „ 31st, 8.5 p.m.
Sunrise June 1st, 3.49 a.m.

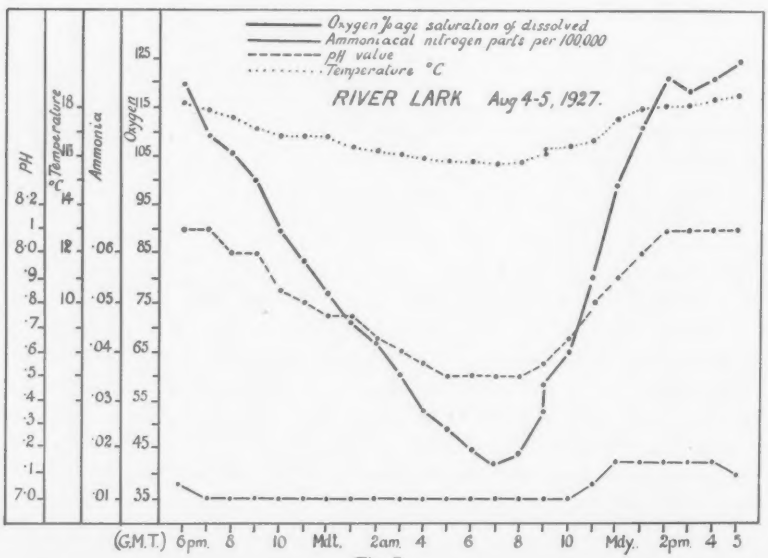

Fig. 7.

Table X. *River Itchen. July 5th–6th*, 1927. *Series IV.*

Time G.M.T.	Temp. °C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
9 a.m.	12·6	1·135	105	0·015–0·02	7·85	Thunderstorm 5.30–6.30 a.m.
10 „	13·1	1·151	108	0·005	7·9	Dull. Occasional sun
11 „	13·1	1·105	103	0·005	7·9	Dull. Raining. Windy
Midday	13·5	1·221	115	0·005	7·9	Dull. Fine
1 p.m.	13·6	1·147	108	0·005	7·9	„ „
2 „	14·1	1·271	121	0·005	7·95	„ „
3 „	15·0	1·217	118	0·005	8·0	Brighter.
4 „	15·0	1·203	117	0·005	8·0	„ Little sun
5 „	15·0	1·200	116	0·005	8·0	Sunshine
6 „	15·1	1·167	113	0·005	8·0	„
7 „	14·9	1·122	109	0·005	8·0	„
8 „	14·0	0·998	95	0·005	7·85	„ Sun low
9 „	13·0	0·923	86	0·005	7·7	Dusk. Fine. Moon shining
10 „	12·5	0·932	86	0·005	7·7	Clear. „ „
11 „	12·0	0·857	79	0·005	7·6	„ „
Midnight	11·8	0·880	80	0·005	7·5	Slight cloud. Moonset
1 a.m.	11·6	0·825	74	0·005	7·5	Cloudy. Sky just lightening
2 „	11·5	0·838	76	0·005	7·5	Daybreak. Some cloud. Fine
3 „	11·1	0·807	72	0·005	7·5	Dawn. Some cloud. Fine
4 „	11·0	0·838	76	0·005	7·5	Daylight. Fine. Clear
5 „	10·7	0·893	79	0·005	7·5	Sunshine
6 „	10·9	0·919	82	0·005	7·6	„
7 „	11·2	1·026	92	0·005	7·7	„
8 „	12·0	1·154	106	0·005	7·8	„ Wind rising
9 „	12·6	1·177	109	0·005	7·9	Dull. Fine

Sunrise July 5th, 3.49 a.m.
Sunset „ 5th, 8.19 p.m.
Sunrise „ 6th, 3.50 a.m.

It would appear that the oxygen exuded from diatoms is more likely to produce a supersaturated solution than is that evolved from the higher green plants which tends to escape in bubbles from their relatively large stomata.

The minimum oxygen values in May, June and August are considerably lower than those of March and April, and it is possible that the larger green plants present during the summer (which were absent in March and April), by increased respiration, have caused this decrease in oxygen during the night. This is confirmed by the slightly lower p_H values obtained during the nights of May, June and August compared with those of March and April. An added factor to the progressive decrease in value of the oxygen minima may be the increased oxygen-consuming power of the river bed and vegetation due to the seasonal increase in temperature.

Nitrogen curves.

As in Series I and II on the River Itchen, no diurnal variation of ammoniacal nitrogen was recorded in Series III, IV and V.

On the River Lark the ammoniacal nitrogen curves of May and June are in general similar in structure to those of March and April, although considerable differences were obtained in the times of the maximum periods. The maximum periods of March and April were from 2–5 a.m. and from

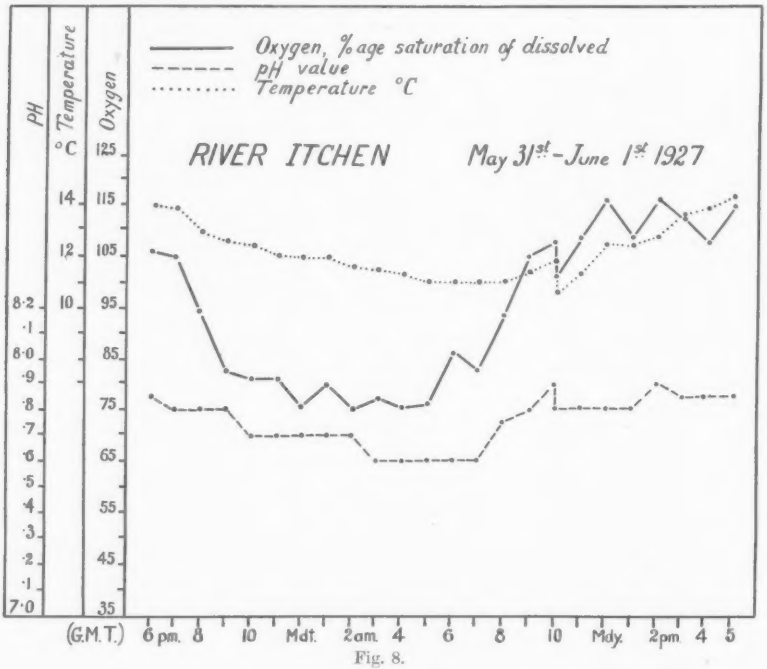
Oxygen, % age saturation of dissolved
pH value
Temperature °C
RIVER ITCHEN
May 31st - June 1st 1927
pH
°C Temperature
Oxygen
(G.M.T.) 6 pm. 8 10 Mdt. 2am. 4 6 8 10 Mdy. 2pm. 4 5

Fig. 8.

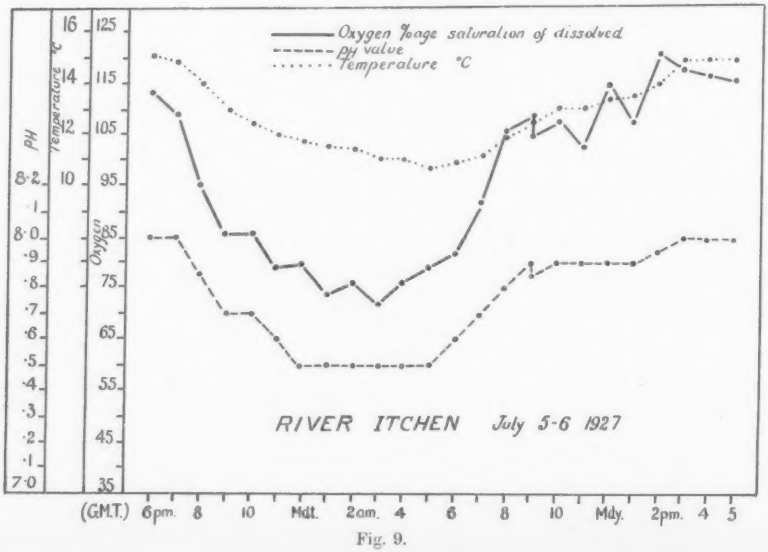
Oxygen % age saturation of dissolved
pH value
Temperature °C
RIVER ITCHEN
July 5-6 1927
pH
Temperature °C
Oxygen
(G.M.T.) 6pm. 8 10 Mdt. 2am. 4 6 8 10 Mdy. 2pm. 4 5

Fig. 9.

Table XI. *River Itchen. July 27th–28th, 1927. Series V.*

Time G.M.T.	Temp. °C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
9 a.m.	12·8	1·060	99	0·005	7·8	Much rain 4–6 a.m. Bright occasional sun. River turbid
10 „	13·5	1·122	106	0·005	7·9	Cloudy. Windy
11 „	13·9	1·128	107	0·005	7·9	Sunshine. Water clearing
Midday	14·7	1·241	119	0·005	8·0	Cloudy. Windy. Water clear
1 p.m.	15·4	1·223	120	0·005	8·1	Sunshine. Windy
2 „	16·0	1·270	126	0·005	8·2	Cloudy. „
3 „	15·9	1·1825	117	0·005	8·2	„ „
4 „	15·5	1·193	117	0·01	8·2	Dull. Very rough
5 „	15·0	1·107	107	0·005	8·0	„ Windy
6 „	14·2	1·067	102	0·005	7·9–8·0	„ Raining 5–6 p.m. Windy
7 „	13·6	0·970	91	0·005	7·9	„ „ „
8 „	13·1	0·863	81	0·005	7·8	Fine. Dull. Windy
9 „	12·9	0·821	77	0·005	7·7	Almost dark. Windy. Cloudy
10 „	12·5	0·833	75	0·005	7·6–7·7	Dark. Windy. Raining
11 „	12·1	0·808	74	0·005	7·6	Dark. Wind dropped. Raining
Midnight	12·0	0·784	72	0·005	7·5	Wind rising. Fine. Cloudy
1 a.m.	12·0	0·789	72	0·005	7·5	Some wind. „ „
2 „	12·0	0·801	73	0·005	7·5	Rain 1.15 a.m. „ „
3 „	12·0	0·760	68	0·005	7·5	Sky lightening. Less wind
4 „	11·8	0·801	73	0·005	7·4–7·5	Dawn. Fine. Some cloud
5 „	11·9	0·793	73	0·005	7·5	Daylight. Cloudy
6 „	12·1	0·834	77	0·005	7·6	Dull. Fine. Some wind
7 „	12·7	0·931	86	0·005	7·7	„ „ Sunny intervals
8 „	13·3	1·106	104	0·005	7·9	„ „
9 „	13·4	1·234	116	0·005	7·9–8·0	„ „

Sunrise July 27th, 4.14 a.m.
Sunset „ 27th, 7.58 p.m.
Sunrise „ 28th, 4.16 a.m.

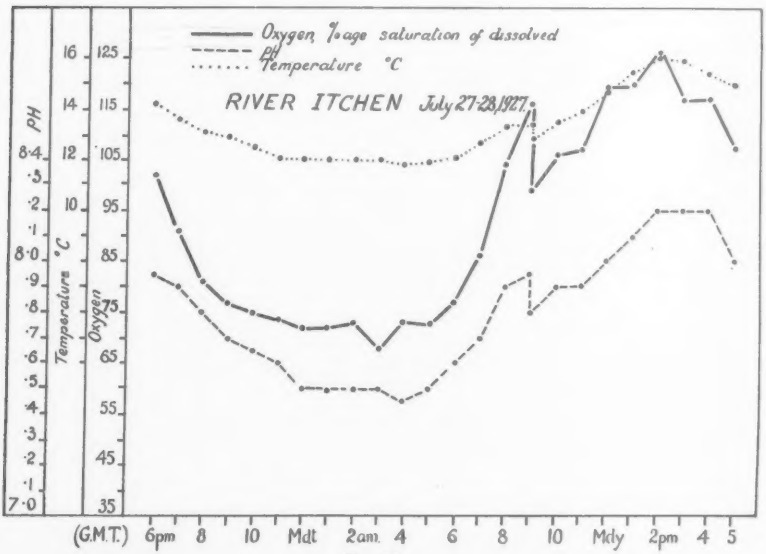
Oxygen % age saturation of dissolved
pH
Temperature °C
RIVER ITCHEN July 27-28, 1927
pH
Temperature °C
Oxygen
(G.M.T.) 6pm 8 10 Mdt 2am 4 6 8 10 Mdy 2pm 4 5

Fig. 10.

Table XII.

Date	% saturation of dissolved oxygen		p_H value		Ammoniacal nitrogen		Temperature °C.		Weather conditions	Sunrise a.m.	No. of days from June 21st	Plants
	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.				
March 30th–31st	3 p.m.	1–7 a.m.	2–7 p.m.	2–9 a.m.	2–5 a.m.	3–6 p.m.	3–4 p.m.	5 a.m.				Submerged and littoral plants just appearing. Diatoms abundant
	157	65	7·9–8·0	7·6–7·7	0·16	0·01	10·0	6·75	Bright	5·40	82	
April 27th–28th	3–4 p.m.	3–6 a.m.	2–6 p.m.	4–7 a.m.	3–9 a.m.	4–11 p.m.	6 p.m.	5–7 a.m.				Submerged and littoral plants 1 ft. in height. Diatoms abundant. Green algae appearing
	178	57	8·0	7·5–7·6	0·16	0·02	11·5	7	Sunny	4·40	55	
May 18th–19th	4–5 p.m.	5–7 a.m.	4–8 p.m.	3–7 a.m.	6 a.m.–midday	6–10 p.m.	5 p.m.	7–8 a.m.				Submerged plants at height of growth. Diatoms on decrease. Green algae dominant
	181	36	8·0	7·5	0·2	0·01–0·015	15·4	10·8	Sunny	4·5	35	
June 28th–29th	3–4 p.m.	6 a.m.	5–9 p.m.	5–8 a.m.	6–10 a.m.	6 p.m.–midnight	3 p.m.	8–9 a.m.				Submerged plants at height of growth. Cut June 22nd. Few diatoms. Some green algae
	111	37	7·9–8·0	7·4–7·5	0·18	0·01	14·5	12·7	Dull and rain	3·40	8	
June 30th	5–7 p.m.	—	—	—	—	—	6 p.m.	—	Afternoon more bright than June 29th			
	110	—	—	—	—	—	16·2	—				
August 4th–5th	4–5 p.m.	7 a.m.	2–7 p.m.	5–8 a.m.	1–4 p.m.	7 p.m.–10 a.m.	5 p.m.	7 a.m.				Submerged plants at height of growth. Some green algae
	124	42	8·0–8·2	7·5	0·015–0·02	0·01	18·4	15·7	Sunny	4·20	44	

3–9 a.m. respectively, whereas those of May and June were from 6 a.m.–midday and from 6–10 a.m. In this respect, some slight relation is shown between the curves of ammoniacal nitrogen and temperature, and it would appear that the higher afternoon temperature of May over June corresponds to an increased and later production of ammonia in May compared to that of June.

A remarkable difference was noted in the ammoniacal nitrogen curve obtained on August 4th–5th—in this case only small differences were obtained throughout the 24 hours, and the maximum period took place between 1 and 4 p.m.

It is difficult to account for this difference between the August curve and those of the previous months, but it is most probable that the river flora and the condition of the river bed are the dominant factors in the disappearance of the high nitrogen values obtained in Series I–IV.

p_H curves.

The May, June and August curves were similar in structure to those of March and April, and in general again follow the oxygen curves.

Temperature curves.

All the temperature curves show the expected diurnal similarity, but over different ranges.

VEGETATION OF THE RIVERS.

A detailed account is given below of the vegetation on the Rivers Lark and Itchen at the times of the various experiments.

The River Lark. For 2 miles above the point of sampling at Icklingham the river is slow flowing and moderately silted. In the summer there is a broad margin of erect littoral plants, mostly *Sparganium erectum* and *Glyceria aquatica*. The submerged plants are sufficiently abundant to hold up the water, causing flooding of the adjoining land even at dry periods. A mile above the point of sampling, the dominant plants are *Potamogeton lucens* and *P. pectinatus*, while immediately above the station, where the water is somewhat deeper, are *Sagittaria sagittifolia* and *Sparganium simplex*. The following is a full account of the vegetation at the various dates of the experiments.

March 30th. The submerged plants were just appearing above the mud and were about 2 inches high. The littoral plants were also just appearing through the mud and previous year's debris.

The diatoms were very abundant, and the figures from deposits on glass slides are given below.

March 9th to 23rd	(1)	10 mg./cm.^{2}
	(2)	15 ,,
March 23rd to April 7th	(1)	73 ,,
	(2)	70 ,,

Dominant organisms: *Synedra ulna* (about 50 %), *Nitzschia acicularis*, *Navicula Reinhartii*, *N. viridula*.

April 27th. The submerged and littoral plants were about 1 foot in height but vigorous growth had not commenced. The diatoms were still abundant, but the proportions of species were not identical.

Deposits:	April 7th to 21st	(1) 47 mg./cm.^{2}
	April 21st to May 5th	(2) 55 ,,

Dominant organisms:

(1) *Synedra ulna* 40 %, *Gomphonema olivaceum* 35 %, *Nitzschia acicularis*, *Diatoma vulgare*, *Melosira varians*.

(2) *Synedra ulna* 40 %, *Ulothrix zonata* 30 %, *Stigeoclonium tenue*, *Gomphonema olivaceum*, *Nitzschia acicularis*, *Amphora minuta*.

A point of note in Sample (2) is the presence of two filamentous green algae among the diatoms.

May 18th. The submerged vegetation had now reached its most vigorous stage of growth and was beginning to hold up the water. Just above the sampling station there was a considerable amount of *Sparganium simplex* (a grass-like plant) but the dominant plants were *Potamogeton lucens* and *P. pectinatus*, both of which were overgrown with *Cladophora glomerata*, a filamentous green alga.

Diatom deposits: May 14th to 30th, 45 mg./cm.^{2}

Dominant organisms: *Ulothrix zonata* 70 %, *Synedra ulna*, *Cymbella gastroides*, *Amphora minuta*.

The diatoms were definitely on the decrease and the filamentous green algae were dominant.

June 29th. Towards the end of May the weeds grew very rapidly, causing flooding of the adjoining fields. On June 22nd weed cutting took place at Icklingham, so that on June 29th a normal amount of vegetation was present, chiefly *Potamogeton lucens* and *Sparganium simplex*. Two miles above the sampling station the littoral plants were fully grown.

Diatom deposits:

	May 30th to June 16th	(1) 1·2 mg./cm.^{2}
		(2) 2·3 ,,
	June 16th to 30th	(1) 1·2 ,,
		(2) 2·4 ,,

Dominant organisms: *Chlorococcum infusionum* and *Protoderma viride*; the deposit consisted of 80 % of *Protococcales*. There were few diatoms; *Amphora minuta*, *Synedra ulna*, *Cymbella gastroides*.

August 3rd. The submerged plants were very abundant, and there was more *Sparganium simplex* than in June. *Cladophora glomerata* and *Potamogeton pectinatus* were present in smaller quantity.

Deposits:	July 1st to 18th	(1) 1·1 mg./cm.^{2}
		(2) 0·7 ,,
	July 18th to August 3rd	(1) 1·4 ,,
		(2) 1·2 ,,

Dominant organisms: *Protoderma viride* and *Chlorococcum infusionum*.

The River Itchen. The Itchen at the Alresford Research Station is a swift stream and the dominant plants are *Sium angustifolium* and *Ranunculus penicillatus*, although immediately above the weir where the samples are taken is a short silted stretch (about 50 yards) with *Callitriche stagnalis* and *Potamogeton densus* in some quantity. Littoral plants are scarce and consist of plants of the habit of *Nasturtium officinale* (water-cress) and *Myosotis palustris* (forget-me-not).

The most remarkable feature of the phanerogamic vegetation is that it persists throughout the year, the area of green plants in the water being not much less in December than in July. The area occupied by the plants is also small in comparison with that in the Lark. The increase in the vegetation therefore in the period considered may be taken as small.

The micro-flora. No direct measurements or systematic collections have been made of the diatoms on the Itchen this year but from previous collections the general seasonal variation is the same as for the Lark, though the quantities are not so large. There is a maximum growth of diatoms in March and April, the dominant organisms being *Synedra ulna*, *Gomphonema olivaceum* and *Amphora minuta*. These decrease in May and are followed by the appearance, as small tufts on all the stones, of the filamentous algae, *Vaucheria* sp. and *Cladophora* sp. At the end of August, these had disappeared and all the micro-flora there was consisted of a few diatoms, *Synedra*, *Gyrosigma*, etc. and *Batrachospermum* in comparatively small quantity.

In conclusion the authors wish again to thank Sir R. Robertson, the Government Chemist, and the Ministry of Agriculture and Fisheries, for permission to publish these results.

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